Aromatic Nitrogen Mustards

Thesis

AROMATIC NITROGEN MUSTARDS

Thesis submitted by

MARIA TERESA TROINA PAMPLONA

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Abstract

This work was stimulated by the search for more potent drug/prodrug combinations in the field of the aromatic nitrogen mustards, for potential used in antibody directed enzyme prodrug therapy.

4-Acyl prodrugs of *N,N*-bis(2-chloroethyl)-4-amino-aniline, possible substrates for the bacterial enzyme acylarylamidase E.C. 3.5.1.13, were synthesised. Attention was focus on 4-*N*'-acetyl and 4-*N*'-trifluoroacetyl compounds. The relative reactivity towards hydrolysis of the prodrug and drug was examined at pH 7.4 and 37°C by HPLC. The hydrolysis of the prodrugs at different pH (2, and 12/13) was also examined and the products identified and quantitated.

The leaving group effect on stability and reactivity was also investigated for a series of simple aniline mustards of the type C_6H_4-N($CH_2CH_2X)_2$ with X-groups other than chloro, by comparing their hydrolysis behaviour in aqueous DMSO at 37°C and pH 7 - 9.

To advance the search for new prodrug structures, the N'-acyl derivative of *N*-(4-nitrophenyl)-4'-aminobenzyl carbamate was synthesised and evaluated as a possible substrate for the acylarylamidase enzyme. The hydrolysis of the 4-N'-acyl compound to its 4-amino parent was briefly examined at pH 2-12 and 25°C. The 4-N'-acyl compound proved to be relatively stable whereas the *N*-(4-nitrophenyl)-4'-aminobenzyl carbamate proved to be very reactive spontaneously decomposing to 4-nitro aniline and other identified products. Synthesis of the 4-amino parent was difficult but it was obtained from the protected 4'-amino carbamate of general formula 4-(G-NH)-C_6H_4-CH_2-O-C(O)-NH-C_6H_4-Z [Z=nitro instead of 4-(*N,N*-bis-2-chloroethylamino)phenyl] by chemical removal of the protecting group G. Several protection groups G were investigated and the best conditions to remove them established.

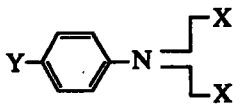
Abbreviations

ABq	AB quartet
Ac	acetyl ($CH_3(CO)$-)
ACN	acetonitrile
ADEPT	antibody directed enzyme prodrug therapy
aqu	aqueous
anhyd	anhydrous
Ar	aryl
t-BOC	*t*-butoxycarbonyl ($(CH_3)_3O(CO)$-)
br	broad
Bu	butyl
CBZ	benzyloxycarbonyl ($C_6H_5CH_2O(CO)$-)
conc	concentration
DCM	dichloromethane
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
EI	electron impact
Et	ethyl
Ether	diethyl ether
EtOAc	ethyl acetate
IEC	ion exchange chromatography
FAB	fast atom bombardment
G	protective group
GC	gas chromatography
GC-MS	gas chromatography-mass spectroscopy
h	hour
HPLC	high performance liquid chromatography
m	minute
Me	methyl

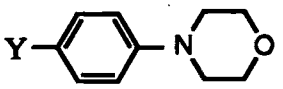
MS	mass spectrometry
Nu	nucleophile
Ph	phenyl
Pr	propyl
Py	pyridine
sat	saturated
THF	tetrahydrofuran
Tr	trityl ($(C_6H_5)_3C$-)
Ts	*p*-toluenesulfonyl (*p*-$CH_3C_6H_4SO_2$-)
TsOH	*p*-toluenesulfonic acid
λ	wavelength
ε	molar absorptivity

Structural Formulae

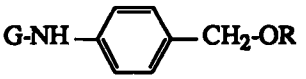
The Structural Formulae of mustards, carbamates and related compounds synthesised in this thesis are represented below:



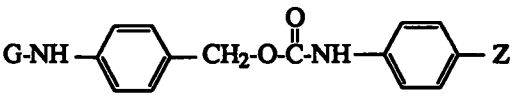
Y	X	Compound index nº
H	Cl	(5)
H	OH	(37)
H	OSO_2CH_3	(38)
H	$O(CO)CH_3$	(39)
H	SCN	(40)
H	ONO_2	(41)
H	OTs	(44)
NO	OH	(50)
NO	OSO_2CH_3	(51)
NO	Cl	(52)
NO	ONO_2	(53)
NH_2	Cl	(10)
NH_3^+ Cl^-	Cl	(54)
NH_2	OSO_2CH_3	(55)
NH_3^+ $^-OSO_2CH_3$	OSO_2CH_3	(56)
NH_2	OH	(66)
$CH_3(CO)NH$	Cl	(11)
$CH_3(CO)NH$	OSO_2CH_3	(57)
$CH_3(CO)NH$	$CH_3(CO)O$	(58)
$CH_3(CO)NH$	OH	(59)
$CF_3(CO)NH$	Cl	(12)
$CF_3(CO)NH$	OH	(65)



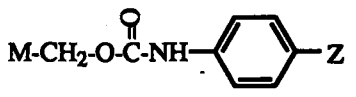
Y	Compound index nº
$CH_3(CO)NH$	(60)
NH_2	(61) *a
H	(45) *a



G	R	Compound index nº
H	H	(80)*a
$(CO)OCH_2C_6H_5$	H	(85)
$C(C_6H_5)_3$	H	(91)
$(CO)OC(CH_3)_3$	H	(93)
$(CO)CH_3$	$(CO)CH_3$	(95)
$(CO)CH_3$	H	(96)



G	Z	Compound index nº
H	NO_2	(74)
$(CO)OCH_2C_6H_5$	NO_2	(75)
$(CO)OCH_2C_6H_5$	CF_3	(76)
$C(C_6H_5)_3$	NO_2	(77)
$(CO)OC(CH_3)_3$	NO_2	(78)
$(CO)CH_3$	NO_2	(26)
$(C_6H_2(NO_2)_3O)(H_2)$	NO_2	(94)



M	Z	Compound index nº
$4\text{-}NO_2(C_6H_4)$	H	(104)
C_6H_5	CH_3	(112)
CH_3	NO_2	(113)

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Chapter 1

Introduction

1.1 An overview of the cancer disease

Cancer is a collective term for over a hundred types of malignant tumours. Malignant tumours are defined as a group of abnormally proliferating cells that can arise in any part of the body and become invasive; that is, they can spread from their sites of origin. If these proliferating cells remain strictly local and non-invasive, they are called benign tumours.[1]

Cancer cells arise from a single cell that has undergone permanent DNA changes. The result of this is that the daughter cells become relatively undifferentiated and lose their cell-cell contact inhibition. Repeated mitosis of these daughter cells then occurs at a much faster rate than those of the surrounding normal cells. There are two main changes in a cancer cell. The first change is of a regulatory nature. The multiplication of the cancer cell does not obey the normal regulatory mechanisms of the organism and it is in a continuous multiplication process. The second change of the cancer cell deals with its relation with the neighbouring cells. Thus, normal cells are limited to specific organs whereas cancer cells can invade other tissues. In addition, they can migrate through the blood or the lymphatic system to establish new colonies of cells in other, distant organs. This process is called metastasis and is responsible for the highly lethal nature of cancer.

Many factors are believed to be involved in the formation of most tumours. A wide variety of agents can act on the normal cells and transform them into malignant cells. The agents which contribute to carcinogenesis include: chemicals,[2] physical agents [3] (eg. ionizing and non-ionizing radiation), and biological agents (viruses,[4] hormones [5]).

Certain differences have been observed between normal and malignant cells. For example, malignant cells often have greater free radical character,[6] lower pH,[7] tumour associated antigens,[8] tumour-produced hormone peptides,[8] higher biowater content,[9] higher potassium ion and lower calcium ion concentrations,[10] different potassium isotope ratios,[10]

and larger amounts of methylated nucleosides.[11] Further, some types of tumour cells (*e.g.* leukemia cells) require an external source of L-asparagine whereas normal cells do not.[12]

Until recently, the treatment of non-disseminated solid tumours was performed by surgery and irradiation. Both methods can be considered to be relatively tumour selective, without significantly harming the rest of the body. If, however, the tumour was disseminated to various organ sites, the metastases could be treated by chemotherapy and immunotherapy only. Chemotherapy is most effective when the number of malignant cells is small. It has considerable side effects due to the lack of specificity of action and the induction of resistance. Immunotherapy alone has a limited tumour spectrum. By using a combination of two or more of these methods, cure rates of certain cancers have been greatly improved. It is often necessary to employ surgical treatment followed by radiotherapy to reduce the tumour size to a level that can be effectively coped with by chemotherapy, which, in turn, would reduce the remaining tumour cells to a small enough number to be completely destroyed by the host immunologic defences.

Recently, some authors refer to the use of chemotherapy alone, performed in a more selective way, in the treatment of some solid tumours.[13] The purpose of the present work is to give some contribution to this goal.

1.2 Aromatic nitrogen mustards used in cancer chemotherapy

Certain drugs inhibit cell division or selectively destroy reproducing cells without permanent injury to non-reproducing cells. The treatment of cancers with drugs of this type is a branch of chemotherapy.

Most drugs in current use inhibit cell division by interfering with either the synthesis or the metabolism of nucleic acids, or with cell mitosis.[14] None of the anti-cancer drugs discovered so far is capable of absolute discrimination between malignant cells and healthy normal cells. The interference with the replication of normal cells, particularly those undergoing fast division as in the case of bone marrow and intestinal mucosa, leads to poor selectivity *i.e.* there is toxicity towards normal as well as cancerous tissue. A similar problem occurs in the radiation treatment of cancer. Thus, such anti-tumour agents produce side effects ranging from nausea and hair loss to suppression of the immune system.

Other problems associated with conventional drug treatment arise because the cancer may initially respond well to the treatment, but then develop a resistant subset of cancerous cells. Furthermore, some drugs fail to penetrate a tumour because of an inadequate blood supply or changes by mutation rendering the cell membrane impermeable to the drug.

The cancers which have responded best to chemotherapy are those where cell division is rapid as in choriocarcinoma (a rare form of cancer originating in the foetal membranes) and acute childhood leukaemia. In contrast, most human cancers such as lung, intestinal and breast cancer, do not respond well to this treatment because of their slower division.[15]

Drugs used for cancer therapy can be categorised by their chemical class, mode of action or origin, *i.e.* whether derived from synthetic or natural sources. Such classes include alkylating agents, platinum derivatives, antimetabolites and antihormonal agents as well as numerous others such as plant products (vincristine, vinblastine and the podophyllotoxins), and antibiotics (Bleomycin and Adriamycin).[15,16]

Alkylating agents were the earliest and most successful anticancer drugs and have been the most systematically investigated. They include the nitrogen mustards (the largest category), aziridines, methanesulphonates and epoxides. All react with various cellular nucleophiles such as hydroxy, amino, mercapto, or imidazole groups of proteins and nucleic acids. However, it is cross-linking of opposite DNA strands by bifunctional alkylation (preventing separation of the two strands essential for cell division) which relates to their chemotherapeutic properties.[17-19] The DNA sites involved in alkylation reactions are preferentially the N7 guanine residue in a reaction dominated by the molecular electrostatic potential of the DNA site (thus, reaction occurs preferentially in the central runs of contiguous guanines where this potential is most negative [20]). It is also possible that alkylation of heterocyclic bases in nucleotides takes place by initial phosphate esterification followed by alkyl group transfer from oxygen to nitrogen.[21-24]

The alkylating agents can have three main biological effects on cells. First, a cytostatic effect (low dosage) whereby cell mitosis is delayed or prevented. Secondly, a mutagenic effect (medium dosage) whereby cell division takes place, but the daughter cells are altered with possible development of carcinogenic properties. Thirdly, a cytotoxic effect (high dosage) whereby cells are irreparably damaged and killed.[17]

In 1946, Goodman and Gilman[25] studied the pharmacology of simple aliphatic nitrogen mustard derivatives and noted their effect on dividing cells. The two compounds tested were tris(2-choroethyl)amine **(1)** and bis(2-choroethyl)methylamine **(2)**, better known as mustine. Compound **(2)**, discovered in the course of work on potential war gases, was found to depress the white blood cell count. This suggested use for treating certain leukaemias and **(2)** was the first mustard used clinically to induce remission in malignant lymphomas.[25] The term 'nitrogen mustard' indicates its isosteric relationship to mustard gas **(3)**.

The chemical reactivity of the nitrogen mustards depends on cleavage of the halogen atom X from the molecule. This reaction is facilitated by the electron releasing capability of the N-atom. Thus, by attaching the nitrogen mustard function to an aromatic ring, it is possible to obtain compounds less reactive than the aliphatic analogues. In order to obtain mild-acting agents, in 1948, Haddow and Ross [26] began the study of the aromatic nitrogen mustards with the general formula $Y\text{-}C_6H_4\text{-}N(CH_2CH_2X)_2$ **(4)** (where X was normaly Cl, Br or I). The first compound to be prepared and tested clinically [27] was the simple aniline mustard **(5)**. Subsequently, a large number of related compounds have been prepared and still remain in clinical use in many multi-drug regimens, for example Chlorambucil **(6)** (one of the slowest acting and least toxic of the nitrogen mustards) and Melphalan **(7)** (a phenylalanine mustard, whose L isomer is at least five times more reactive than the D isomer, showing the importance of chirality in biological activity[28]). The aromatic nitrogen mustards (in contrast to their aliphatic counterparts which are highly reactive and vesicant) can be taken orally to reach target sites before extensive dissipation by side reactions.

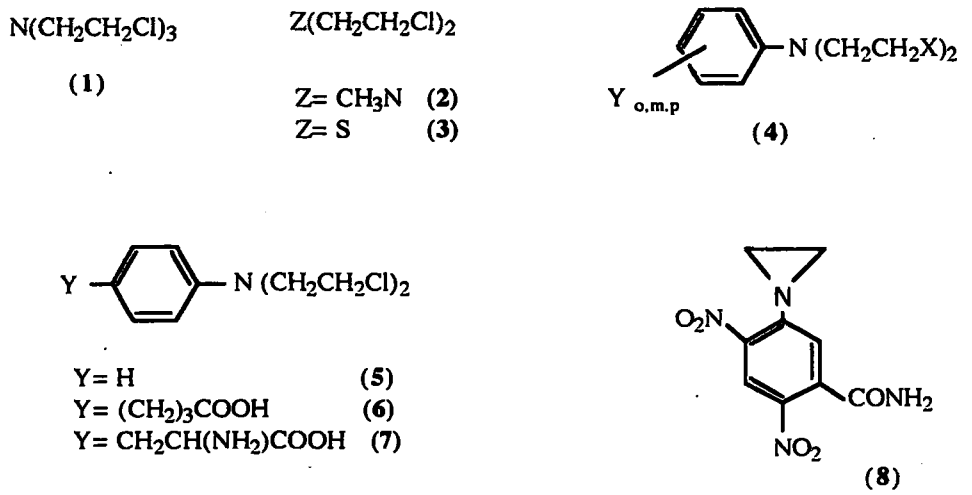


Chart 1 Structures (5) - (8)

Early workers tried several modifications of the mustard structure in order to improve the selective toxicity towards cancer cells. In 1952, Danelli [29] pointed out that drugs could be made more selective by increasing the number of variables affecting drug activity. Variations could be achieved by exploiting differences in chemical reactivity and physical properties (solubility, polarity) of drugs, and differences between normal and malignant cells (pH,[30] enzyme levels, etc). Furthermore, moieties such as amino acids,[28,31] carbohydrates,[32] polypeptides, nucleosides and steroids could be used either to convey or to

inactivate the toxic constituent.[33,34] Examples of drugs where attachment of the alkylating residue to a naturally occuring molecule (*e.g.* sugar, amino-acid) facilitate selective transport into tumour cells include Degranol[35] (a D-manital derivative), Melphalan[28] (a L-phenylalanine derivative) **(7)**, and Phenesterin [36] (a cholesterol ester derivative).

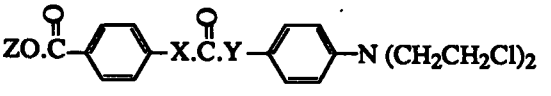
The use of biological moieties to inactivate the toxic constituent of the anti-cancer drug is the principle of latent chemotherapeutic activity. This implies the design of a compound (prodrug) which is inactive but convertible *in vivo* (hydrolysis, reduction, oxidation) into an active form (drug) at the tumour site.

The important observation of Connors *et al.*,[37] that the relatively inert mono functional alkylating agent, 2,4-dinitro-5-ethyleneiminobenzamide (CB1954) **(8)**, had a profound therapeutic effect on the Walker rat carcinoma but showed little or no effect on other cancers, suggested that the Walker carcinoma cells converted **(8)** to a potent cytotoxic agent. This reinforced the principle and potential benefits of drug activation at the site of the cancer. Recently, Roberts *et al.* [38] proposed that **(8)** is converted enzymatically to a difunctional agent which caused DNA-DNA interstrand links.

Both the establishment of the principle of activation of a compound at a specific site and the observation of high levels of the azo reductase enzyme in primary liver tumours, led to the introduction of azomustard prodrugs **(9)** (***Scheme 1.1***). These compounds are deactivated by the conjugated ring system, and therefore show very low toxicity.[39] On reaching the cancer cells, the protected mustards are reduced by azo reductase and converted to the highly toxic parent *N,N*-bis(2-chloroethyl)-4-amino-aniline (phenylenediamine mustard) **(10)**, as outlined in ***Scheme 1.1***. The reactivity of **(10)**, the most common alkylating moiety employed, relates to electron donation by the 4-amino group, which increases the basicity of the tertiary nitrogen atom and facilitates the the formation of the aziridinium ion intermediate. Coupling of this 4-amino group to an electron withdrawing group reduces the electron donation power, thus the adduct formed should be less reactive than the free phenylenediamine mustard. Examples of other prodrugs that could be converted by enzymatic action into the effective drug **(10)** include the *N'*-acylated derivatives (for which the acetyl **(11)** and the trifluoroacetyl **(12)** derivatives, first synthesised by Ross,[40,41] were expected to be hydrolysed by an amidase [40]) and the carbamate (urethane) derivatives **(13)**.[32,42] On hydrolysis of the urethane group and consequent loss of CO_2 (***Scheme 1.1***), the full reactivity of the parent mustard **(10)** becomes available. Incorporation of the electron attracting *N'*-acyl or *N'*-urethane group in the aromatic ring should lower the reactivity of the mustard moiety because it reduces *N*-electron donation. The elaboration of aromatic

nitrogen mustards in these ways was extensively studied by Ross,[41,43,44] Owen,[42,45-47] Warwick [48] and their collegues.

It was suggested by Danielli,[49] and confirmed later by Benn,[32] that tumours develop adaptive enzymes after continuous administration of carbamates. These enzymes decompose the carbamate by fission of an ester or peptide linkage, and this condition is favourable for selective activation, at the tumour site, of carbamate derivatives of both *N,N*-bis(2-chloroethyl)-4-amino-aniline (10) and *N,N*-bis(2-chloroethyl)-4-hydroxy-aniline mustards. In particular, compounds like (16), its methyl ester (17), or the 'reversed' carbamate (18), proved to be of therapeutic interest because they possessed a high degree of selectivity against the Walker 256 tumour with particularly high chemotherapeutic indices (LD_{50}/ED_{90}) of 46, 137 and 17, respectively [47] [the larger the therapeutic index, calculated by dividing the dose which kills 50% of the animals in a group (LD_{50}) by the dose which produces 90% inhibition of tumour growth (ED_{90}), the greater the relative safety]. In comparison, Chlorambucil (6) has a chemotherapeutic index of 12 when measured under the same conditions. For compounds (16) and (18), the carboxylic acid group increased the solubility in aqueous media, overcoming the problem of insolubility conferred both by the mustard and the carbamate functions.



(16) Z = H, X = O, Y = NH

(17) Z = Me, X = O, Y = NH

(18) Z = H, X = NH, Y = O

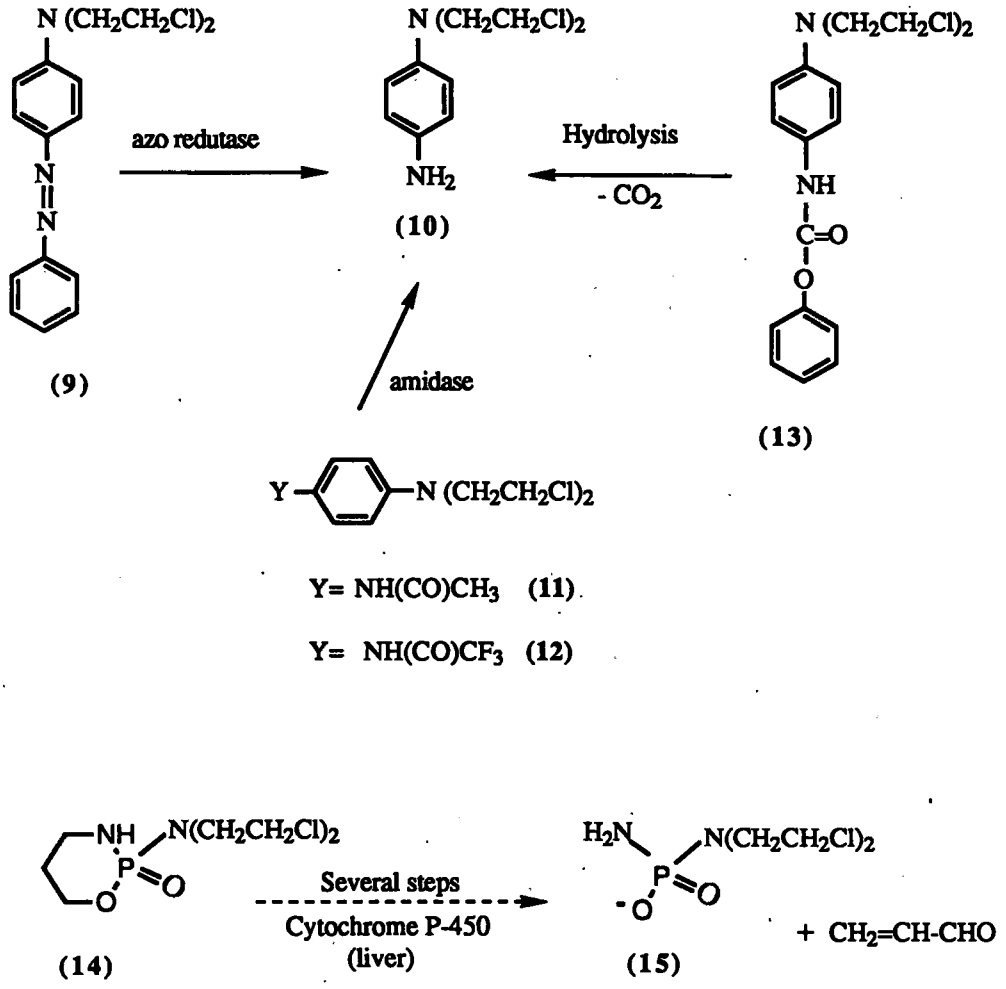
Chart 2 ***Structures (16) - (18)***

In parallel, many phosphamide mustard prodrugs were produced as contenders for hydrolysis by phosphoramidases, reputed to be concentrated in tumour tissues. The most successful of these was Cyclophosphamide (14) [15] in which the electron-withdrawing P=O group prevents cyclization of the nitrogen mustard by decreasing the availability of the nitrogen lone pair. Later, it was shown that activation of (14) was not due to hydrolysis within the tumour, but to liver microsomal oxidation by the cytochrome P-450 system which catalyzed C4-hydroxylation. The resulting 4-hydroxycyclophosphamide undergoes ring

opening to aldophosphamide, followed by β-elimination to generate cytotoxic phosphoramide mustard (15) and acrolein (CH_2=CH-COH) (*Scheme 1.1*).

The principal problem with the prodrug approach was the unfavourable distribution of activating enzymes in human tumours. However, whereas enzymes do not appear to be disposed in a consistent and exploitable fashion in human tumours, some macromolecules viewed as antigenic determinants have shown a more favourable distribution. Consequently, there have been many attempts to increase selectivity by coupling cytotoxic substances to antibodies directed at tumour associated antigens. These have been shown to localise selectively though not exclusively, at cancer sites *in vivo*.[50-55] Monoclonal antibodies armed with drugs, toxins or radioactive isotopes as cytotoxic warheads have been studied, and these constitute the antibody directed therapy.[56,57] The first general strategy was to conjugate the toxic component to the antibody in a single bifunctional molecule.[50] One problem with this approach is heterogeneity in the distribution of the antigens within tumour cells, so that even if an antibody-drug conjugate is internalised by cells expressing the antigen, a substantial proportion of cells fail to express it and so, escape. Also, the slow clearance of antibodies from blood, which may take several days,[58] contributes to cytotoxic effects in normal tissues. Moreover, the direct conjugation of toxin to antibody limits the number of toxic molecules delivered to that of antibodies reaching the tumour. Thus, when cytotoxic substances are coupled to antibodies, time-concentration ratios for tumour and normal tissues tend to be less favourable than is necessary for major therapeutic effect. Yet a further problem is that of host antibody response to foreign protein, which can prevent treatments being repeated.

An improvement to antibody directed therapy (which had demonstrated favourable tumour localisation of the anti-cancer agent, but only limited therapeutic success) was achieved by separating the tumour targeting conjugated enzymes (antibody-enzyme complexes able to activate prodrugs) from the toxic prodrug component, in two different molecules to be administered separately. Such targeting localised the enzyme to either the tumour cell membrane or to extracellular sites around tumour cells. The amplification inherent in the enzyme component of the antibody-enzyme conjugate may compensate for the small amount of conjugate retained in the tumour. This new approach constitutes the principle of Antibody Directed Enzyme Prodrug Therapy (ADEPT).[57]



Scheme 1.1 Some nitrogen mustard drug/prodrug combinations used in chemotherapy

In parallel with the development of the prodrug concept and the antibody directed therapy, another technique known as combination chemotherapy has been used in the treatment of leukemias and lymphomas. This involves the administration of several drugs, simultaneously or sequentially, to preventing the development of resistance and to obtain a synergistic effect.

Other recent work [59-61] to improve the efficacy of aromatic mustards exploits compounds with greater specificity for DNA. This approach should reduce the chance of losing active drug by side reactions with other cell components. Thus, with DNA-affinic carrier molecules (e.g. DNA-intercalating ligands like 9-aminoacridine) attached to the mustard, drug potency is increased, and the pattern of DNA lesions (and their repair) altered to reduce the monoadduct (genotoxic lesions) to cross-link (cytotoxic lesions) ratio.[62]

Work is also proceeding to overcome problems with conventional enzymatic activation of prodrugs. The development of new prodrugs capable of bioactivation only in the absence of oxygen, as happens in large solid tumours where the blood supply is disorganized and inefficient, to give hypoxic regions, is being explored. Both hypoxia-selective antitumour agents based on nitrophenyl mustards [63,64] in which activation relates to the cellular reduction of the nitro group to the amino group, and quinone mustards,[65] are under investigation.

1.3 Antibody directed enzyme therapy (ADEPT)

The concept of ADEPT (Antibody Directed Enzyme Prodrug Therapy) is a more recent approach in the treatment of cancer. It was first demonstrated by Philpott *et al.* [66-68] in 1973 but Bagshawe and his colleagues [56,57,69,70] are primarily responsible for its development and application in 1987, although Senter [71] has made useful contributions. It consists of a two stage treatment where the delivery plus activation and the cytotoxic functions are carried on different molecules. First, the enzyme coupled to an antibody is directed to the corresponding antigen of the tumour cell. This first component combines the selective delivery of the antibody with a capability to generate a cytotoxic agent (drug) from a second subsequently administered component (prodrug). As with intact antibodies, the antibody-enzyme complex achieves the most favourable tumour to non-tumour distribution ratio several days after intravenous injection, by which time it has largely cleared from the blood and normal tissues. Administration of the prodrug at this time constitutes the second stage of the process and should result in the selective generation by enzyme catalysis of the active toxic drug at the tumour sites.

Enzymes chosen for such a role can be non-mammalian in origin and thus lack a human analogue. Ideally, there would be no comparable enzyme in the intestinal flora, although the importance or otherwise of this constraint would depend on the nature of the prodrug and whether it was secreted into the gastrointestinal tract. It is necessary to ensure that the enzyme can be conjugated to an antibody and remain stable and active in the extra-cellular compartment of tumours for relatively long periods.

One requirement for the prodrug is that it should be a compound of low toxicity that can be converted to a highly active cytotoxic substance only by the action of the chosen enzyme. The active product is likely to be a small molecule and therefore highly diffusible through the tumour. A short half life (a few seconds) would avoid toxic effects on normal cell renewal tissues.

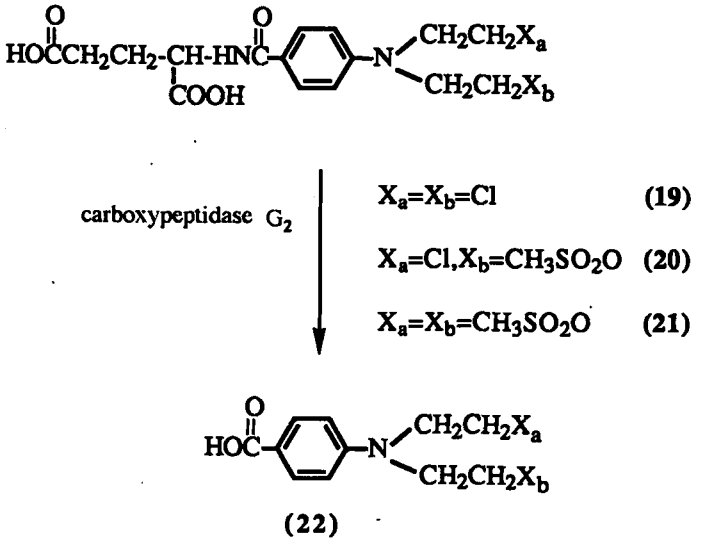
The therapeutic success of ADEPT approach depends on several factors: (a) the stability of the prodrug *in vivo;* (b) the difference in toxicity between prodrug and drug; (c) the molar toxicity of the drug; (d) the pharmacokinetics of the prodrug; (e) the tumour selectivity of the antibody enzyme conjugate; (f) the turnover rate of the enzyme used in the conjugate; (g) the molecular weight and pharmacokinetics of the antibody enzyme conjugate; (h) the percolation and retention of the antibody enzyme conjugate at the tumour site and its elimination kinetics from normal tissues; (i) the concentration of the free unconjugated endogenous enzyme; and finally (j) the immunogenicity of the antibody enzyme conjugate.[72]

To overcome a common problem of rejection associated with the use of an antibody chemically linked to the enzyme of bacterial origin, Bosselet *et al.*[72] recently developed a fusion gene consisting of a humanised tumour selective binding portion and a human lysosomal enzyme, which due to its fully human or humanised building blocks, should have a low immunogenicity in humans. This fusion protein might allow a site specific antibody directed enzyme prodrug therapy *in vivo.*

The multiplicity of non-mammalian enzyme and prodrug combinations suggests that targeted enzyme chemotherapy is a large, new area to explore. Researchers are looking for new antibodies with specificity for human cancers as well as more suitable enzyme prodrug combinations. Monoclonal antibodies and improved techniques in immunohistochemistry allow detailed analysis of binding to tumours and normal tissues. Cell lines provide a viable cell test bed. Human tumour xenografts in nude mice [73] or rats, allow *in vivo* testing of potential antibodies and conjugates for their ability to withstand *in vivo* conditions, and to localise at target sites. Also, antibodies can be tested in humans for their ability to localise in tumours. An antibody or other vector to improve the selectivity of cancer chemotherapeutic agents can only be considered for therapeutic use after its credentials in these preliminary situations have been thoroughly examined.[73]

In 1986 Searle *et al.*[74] reported the first enzyme-antibody conjugate as an anti-tumour agent against choriocarcinoma cells *in vitro.* The enzyme carboxypeptidase G2 (CPG2), a zinc metaloenzyme isolated by Sherwood [75] from the bacteria *Pseudomonas sp.*, was linked to a monoclonal antibody and directed at a subunit of human chorionic gonadotrophin. This enzyme catalysed the hydrolytic cleavage of reduced and non-reduced folates to pteroates and L-glutamate, thereby diminishing the continued external supply of folate, an essential metabolite in DNA synthesis and consequently, in cell replication. Subsequently (1988), carboxypeptidase G2 was used in the first matching antibody-enzyme-prodrug combination, by Bagshawe and co-workers [76] to activate an alkylating agent (mustard (19)) (*Scheme 1.2*) and to eradicate an established human cancer xenograft of

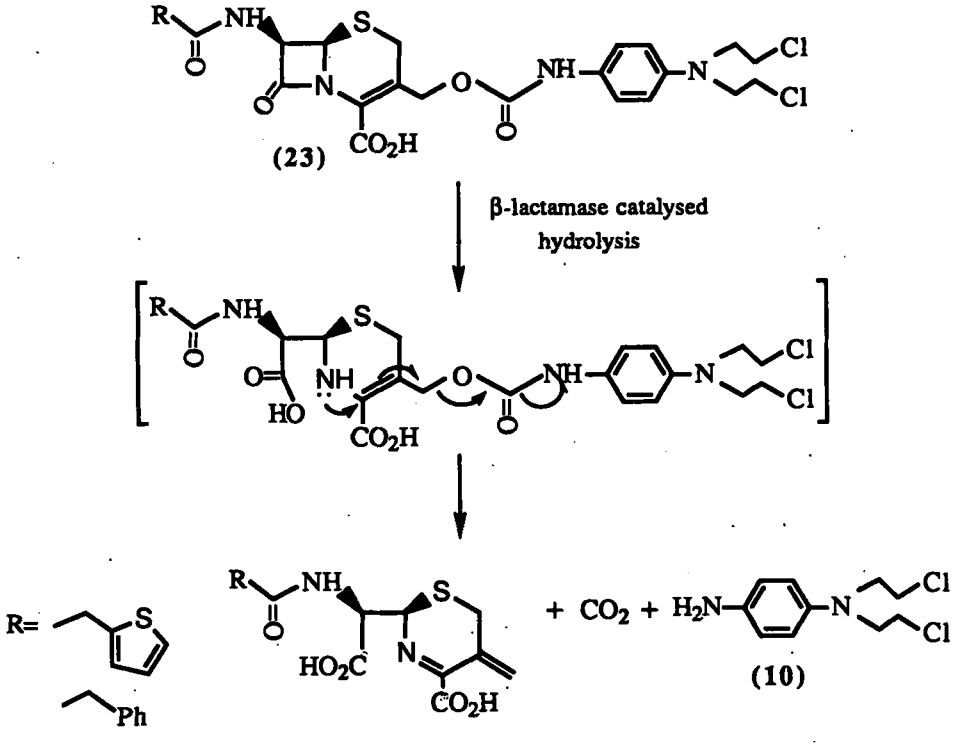
choriocarcinoma. This tumour is resistant to conventional chemotherapy. Carboxypeptidase G2 activated the prodrug by hydrolysis of the amide bond (as in folates) with release of glutamic acid and the alkylating agent (22). The conjugate carboxypeptidase G2-monoclonal antibody, was also examined in nude mice with xenografts of colorectal carcinoma.[77] After time for the conjugate to localise at tumour sites and clear from blood, the prodrug 4- [(*N*,*N*-bis(2-chloroethyl)amino]benzoyl-L-glutamic acid (19) was injected and converted into the active benzoic acid mustard derivative (22) (*Scheme 1.2*). Mustard (22) was about 100-fold more cytotoxic than its glutamated prodrug (19). In the absence of carboxypeptidase G2, the prodrug hydrolysed to active drug very slowly, whereas enzyme and antibody-enzyme conjugate effected rapid hydrolysis. Later, other related compounds [e.g. 4-[*N*-(2-chloroethyl)-*N*-(2-methanesulphonyloxyethyl]amino]benzoyl-L-glutamic acid (20), and 4-[bis-*N*,*N*-(2-methanesulphonyloxyethyl)amino]benzoyl-L-glutamic acid (21)] were shown to be suitable prodrugs for carboxypeptidase G2 with efficacy in two different human tumour cell lines [78,79] and human choriocarcinoma xenografts implanted in nude mice.[80] The first clinical success for ADEPT was achieved in 1991 by the Bagshawe team [13] in the treatment of solid colorectal carcinoma using prodrug (20) with carboxypeptidase G2 linked to a fragment of a murine antibody. These alkylating agents were found to be good candidates for ADEPT in that their cytotoxicity is dose-related and they can be given repeatedly with less induced resistance than other classes of anticancer agents.[81,82]



(22)

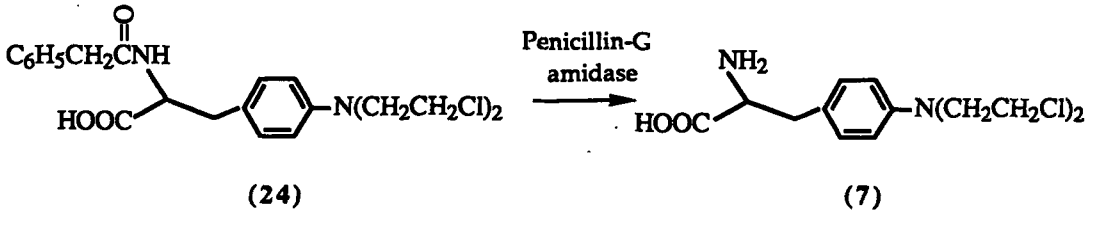
Scheme 1.2 The first mustards used in ADEPT

Recently,[83,84] two other teams have reported that a series of cephalosporin mustard prodrugs (23) activated by a monoclonal antibody-β-lactamase (extracted from *Bacilus cerus* and *Escherichia coli*) can also be used with success in ADEPT. β-Lactamase catalyzed the release of the active *N,N*-bis(2-chloroethyl)-4-amino-aniline (10) [or related compounds - $HN(CH_2CH_2Cl)_2$, $HN(CH_2CHMeCl)_2$, $HN(CH_2CH_2Br)_2$, $HN(CH_2CHMeBr)_2$] through a fragmentation reaction which occurs after the β-lactam ring is hydrolysed (*Scheme 1.3*).



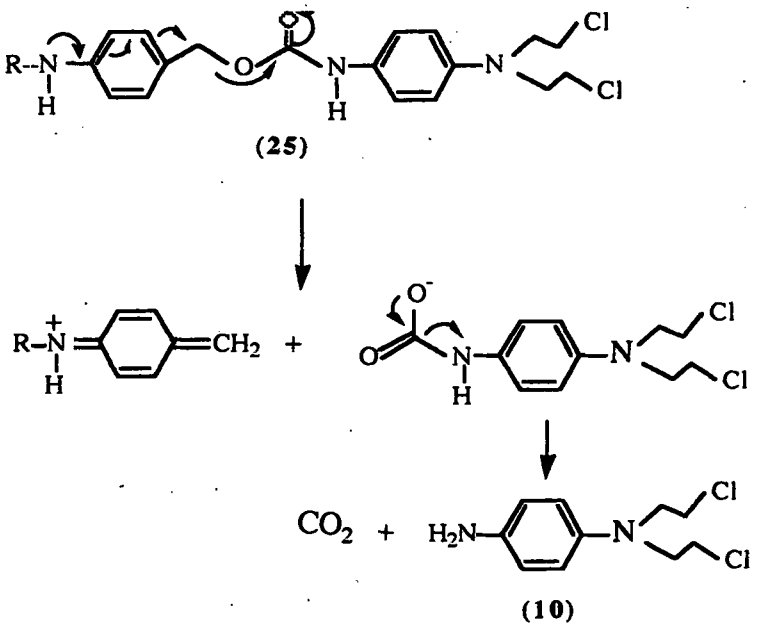
Scheme 1.3 Cephalosporin mustard carbamate prodrug used in ADEPT

A similar success has been reported [85] for the activation of a *N*-phenylacetamido derivative of Melphalan (24) into active Melphalan (7) by a monoclonal antibody-Penicillin-G amidase conjugate (*Scheme 1.4*). This establishes that (24) is a prodrug that can be enzymatically activated to form an active anticancer agent.



Scheme 1.4 Melphalan prodrug used in ADEPT

The work reported by Raison [86] on the reactivity of the quinone methide structure implies that compounds of general structure (25) (containing a carbamate group) should easily decompose with liberation of CO_2 and the active drug (10) (*Scheme 1.5*). With this in mind, our aim was to synthesize a model compound [*N*-acetyl carbamate (26), where the mustard moiety was substituted by the nitro group] and to evaluate its suitability as a substrate for an acyl amidase enzyme and its ability to generate 4-nitro aniline, as suggested by *Scheme 1.5* . Evidence was sought that (26) could be considered [after substitution of the nitro group by a mustard moiety like (10)], a prodrug for ADEPT.



Scheme 1.5 Proposed mechanism for quinone-methide prodrug reactivity

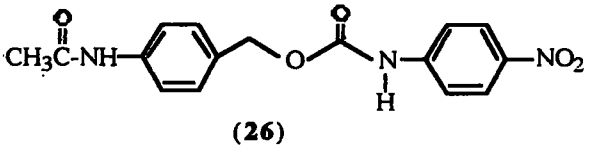
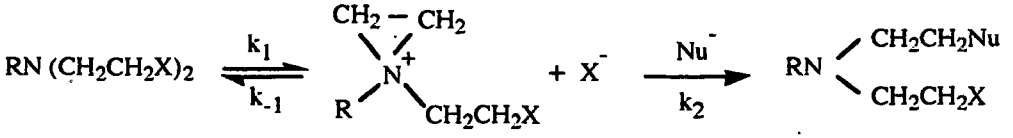


Chart 3

1.4 Alkylation reactions of aromatic nitrogen mustards

1.4.1 Alkylation mechanisms

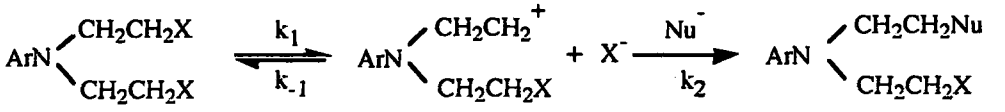
Nitrogen mustards are powerful alkylating agents which reflects their use as antitumour drugs. The mechanism(s) for these reactions have been the subject of considerable debate, but detailed kinetic studies of their reaction with DNA are not available. Claims of S_N1 mechanisms (some involving primary carbocations in solution), S_N2 mechanisms, and internal S_N2 processes wherein nucleophilic nitrogen participation affords an aziridinium ion intermediate have been made. There is some concensus for reaction via an S_N1 mechanism,[87] and that their biological effectiveness relates to the formation of an aziridinium ion intermediate (27),[43,44,88] the formation of which is the rate-determining step. The formation and stability of the aziridinium ion is highly dependent on the basicity of the nitrogen (i.e. the availability of the nitrogen lone-pair electrons). The existence of this aziridinium ion intermediate, first proposed by Everett and Ross, has been demonstrated for aliphatic mustards[89-93] (*Scheme 1.6*), but disputed for the less basic, aromatic mustards.[43,44,94-97] For these Everett and Ross proposed an S_N1 pathway with rate



(27)

Scheme 1.6 The formation of cyclic aziridinium ion intermediate from aliphatic 2-halogenoethylamines

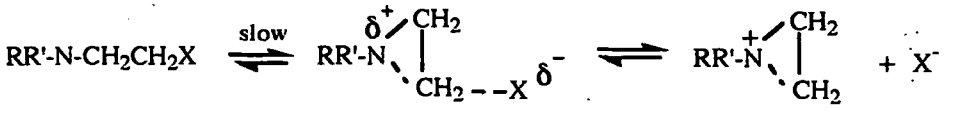
limiting formation of a carbocation intermediate (28) (*Scheme 1.7*).



(28)

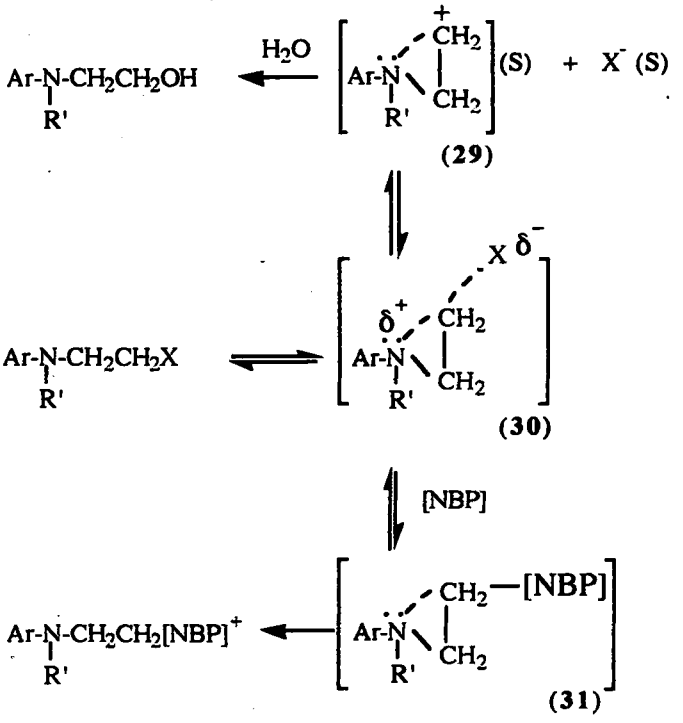
Scheme 1.7 Unimolecular hydrolysis of aryl nitrogen mustards involving a carbonium ion intermediate

Chapman and James [98] criticised this mechanism and suggested an internal bimolecular mechanism in which the unshared electron pair on the β-nitrogen atom cooperates in the decomposition (*Scheme 1.8*). This internal S_N2 mechanism still leads to first order kinetics provided that the first step is rate-determining.



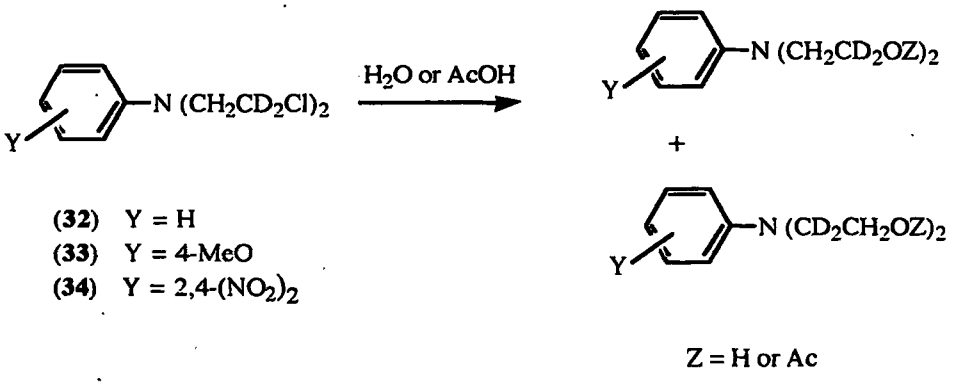
Scheme 1.8 Internal bimolecular mechanism

Bardos *et al.* [88] modified this proposal after comparative studies of the hydrolysis and alkylation of 4'-(4-nitrobenzyl)pyridine (NBP) (*Scheme 1.13*) and suggested that S_N1 hydrolysis and S_N2 alkylation of NBP proceeds as shown in *Scheme 1.9*. According to this mechanism, the high-energy transition state of hydrolysis is a solvated carbocation-nitrogen dipole (29), whose collapse gives the observed product of hydrolysis. The transition state for alkylation is an 'S_N2 complex' (31), consisting of an incipient aziridinium ion partly opened by the nucleophilic NBP reagent. Both hydrolysis and alkylation pass through transition state (30).

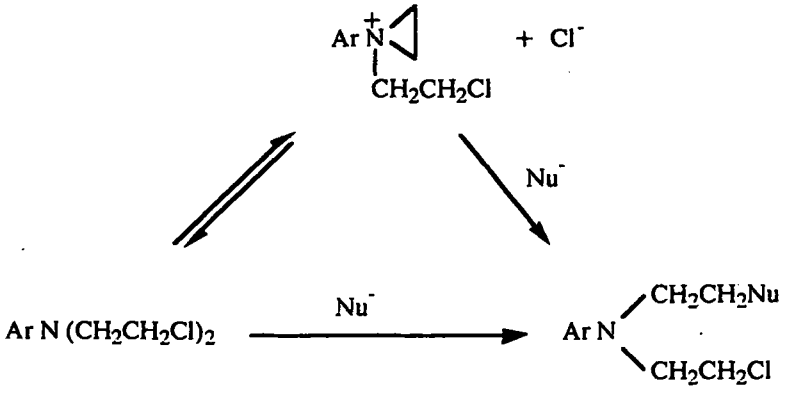


Scheme 1.9 Dual mechanism of nucleophilic displacement for aryl nitrogen mustard involving the same transition state

Reaction via an aziridinium ion intermediate was also supported by Price *et al.*[95] and by Williamson and Witten [94] on kinetic arguments, and by Benn *et al.*[96] who studied substitution reactions of aryl mustards (32) to (34) labelled with deuterium in the 2'-position (*Scheme 1.10*). Scrambling of the deuterium label for both hydrolysis and acetolysis, proved intermediate formation of an aziridinium ion. Treatment with powerful nucleophiles, however, produced no scrambling of the deuterium label, which required direct displacement of Cl^- without intermediacy of the aziridinium ion. The high reactivity of the aziridinium ion (29) compared to an alkyl analogue seems reasonable, and appreciable concentrations are therefore not expected. So, in 1970, Benn *et al.*[96] summarised the available data and concluded that nucleophilic displacement of the side chain halogen of aromatic nitrogen mustards may proceed by one of two competitive pathways (*Scheme 1.11*). The first involving direct S_N2 displacement, the second via the reactive aziridinium ion intermediate. The second pathway is preferred, except for reactions with powerful nucleophiles.



Scheme 1.10 *Scrambling of the deuterium label for both hydrolysis and acetolysis of aryl mustards*

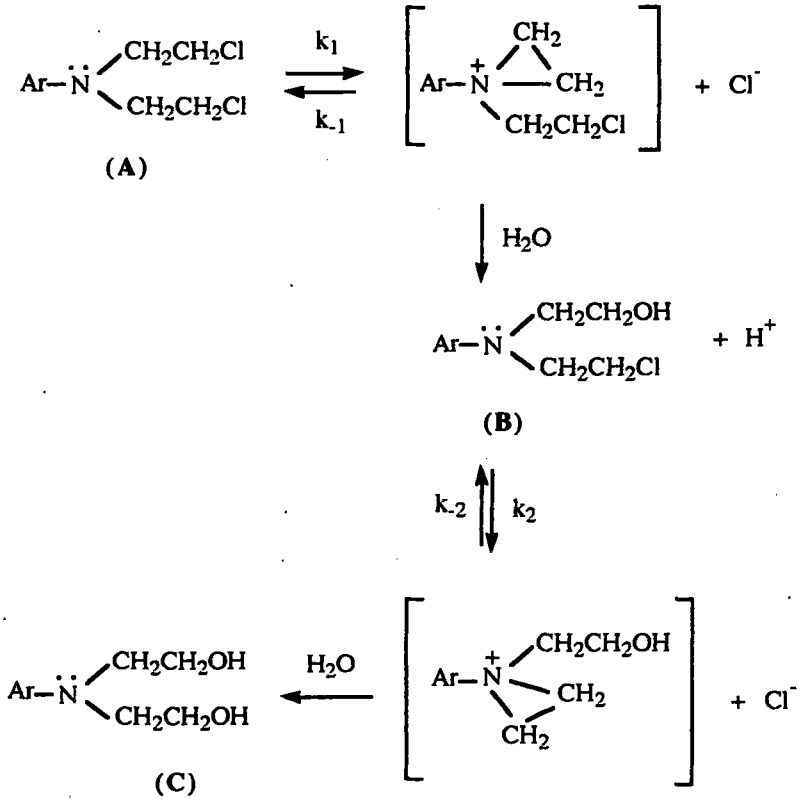


Scheme 1.11 *Proposed mechanism for the nucleophilic displacement of the aryl nitrogen mustard (Benn et al., 1970)*

1.4.2 Kinetics of hydrolysis

It is accepted that bis-2-chloroethyl aromatic nitrogen mustards **(A)** undergo stepwise hydrolysis with release of chloride ion and the formation of an aziridinium ion, which is attacked by water (or other nucleophiles) to yield **(B)**. The same sequence is then repeated for the second 2-chloroethyl group in **(B)** to give the final diol product **(C)** (*Scheme 1.12*).

The hydrolysis of aromatic nitrogen mustards (even the more reactive) in aqueous acetone follows first order kinetics, with the formation of the aziridinium ion being rate--limiting. Factors that slow this step, such as the addition of chloride ion or a non-polar organic solvent, prevent accumulation of the aziridinium ion.



Scheme 1.12 *The two stage process involved in the hydrolysis of mustards*

The integrated rate equations for sequential first-order reactions (equation 1.1) are given by equations 1.2 to 1.5,[99] where A_0 is the initial concentration of **A**, $[A]_t$, $[B]_t$, $[C]_t$ and $[Cl]_t$ are the concentrations of **A**, **B**, **C** and chloride ion, respectively, at time t, and k_1 and k_2 are the *pseudo* first-order rate constants for the first and second steps:

$$\mathbf{A} \xrightarrow{k_1} \mathbf{B} + Cl^- \xrightarrow{k_2} \mathbf{C} + Cl^- \qquad (1.1)$$

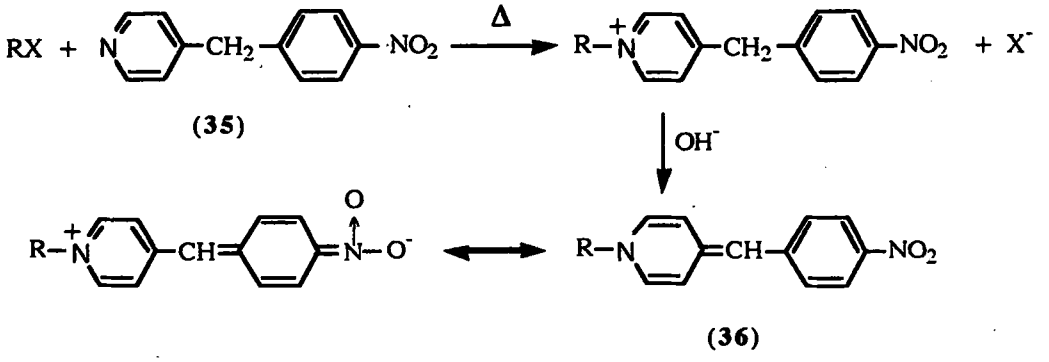
$$[\mathbf{A}]_t = A_0 e^{-k_1 t} \qquad (1.2)$$

$$[\mathbf{B}]_t = A_0 k_1/(k_2-k_1).(e^{-k_1 t} - e^{-k_2 t}) \qquad (1.3)$$

$$[\mathbf{C}]_t = A_0/(k_2-k_1).[k_2(1- e^{-k_1 t}) - k_1(1-e^{-k_2 t})] \equiv A_0-[\mathbf{A}]_t-[\mathbf{B}]_t \qquad (1.4)$$

$$[Cl]_t = [\mathbf{B}]_t + 2[\mathbf{C}]_t \equiv A_0(1-e^{-k_1 t}) + A_0/(k_2-k_1).[k_2(1-e^{-k_1 t}) - k_1(1-e^{-k_2 t})] \qquad (1.5)$$

There has been much controversy over the evaluation of k_1 and k_2 values from experimental measurements. Most early kinetic work was based on indirect determination of unreacted **A** by estimation of released hydrogen[17,43,88,91,100,101] or chloride-ion assays[44,94,102] (using potentiometric,[103,104] conductimetric[105] or ion selective electrodes[106,107]) or reaction with 4'-(4-nitrobenzyl)pyridine (NBP) **(35)**,[88,108,109] to produce a coloured pyridinium salt **(36)** at alkaline pH (*Scheme 1.13*). This last method had a drawback since NBP reacts with all alkylating agents, measuring both intact mustard and the intermediate aziridinium ion. All of these indirect methods give incorrect estimates of mustard stabilities.[110,111] Recent investigations using high-performance liquid chromatography (HPLC) for the hydrolysis of Melphalan [112-114] and Chlorambucil, [115] and the hydrolysis and alkylating reactivity towards NBP and thiourea for 4-anilinoquinolinium aniline mustards,[116,117] gave more useful information on both the kinetics of mustard hydrolyses and the effect of chloride ion. HPLC allows simultaneous quantitation of the substrate-**A** and both the major hydrolysis products, half-mustard-**B** and diol-**C**. Thus, *pseudo* first-order rate constants for the first (k_1) and second (k_2) hydrolysis steps can be calculated from peak areas (*i.e.* concentrations) of the three species.



Scheme 1.13 *Reaction of alkylating agent with 4'-(4-nitrobenzyl)pyridine*

1.5 Quantitative structure-activity relationships for aromatic nitrogen mustards

Quantitative structure-activity relationships (QSAR) have been formulated by several authors (Lien and Tong,[118] Panthananickal *et al.*,[119-121] Bardos *et al.*,[88,122] Niculescu--Duvaz *et al.*,[123-125] and Denny *et al.* [126,127] for a variety of aniline mustards of general formula Y-$C_6H_4N(CH_2CH_2X)_2$ **(4)** where X=Cl, Br or I. Substituent constants for these structure-activity correlations were in large proposed by Hanch and Leo [128-130] and also by Mager [131] and Exner.[132,133] These relationships correlate chemical reactivity [i.e. rates of either hydrolysis or alkylation of 4'-(4-nitrobenzyl)pyridine (NBP)] with biological activity [expressed in multiple ways including toxicity [eg. LD_{50} - molar dose (mol kg^{-1}) required to produce death in 50% of the population], antitumour activity [eg. ED_{90} - molar dose (mol kg^{-1}) to produce 90% reduction of tumour cell population], Hammett substituent parameters (σ), lipophilicity constants (π), steric parameters (I) and mutagenic and carcinogenic activities.[121]

From the work of Bardos and co-workers [88,122] with 4-substituted derivatives of **(4)** (X=Cl, Br or I), equations 1.7 and 1.8, respectively, were formulated for the antitumour activity (ED_{90}) against the solid rat Walker 256 carcinoma and toxicity (LD_{50}).[119] In these equations (and equations 1.9 to 1.13), n = number of compounds analysed; r = correlation coefficient; σ (or σ^- depending on the best fit) = Hammett electronic parameter; I_{Br} (and I_0 in equations 1.9 and 1.10 and I in eqn. 1.12) = indicator variables to define the steric parameters (I_{Br}=0 for X=Cl or I but I_{Br}=1 for X=Br); π = Hansch lipophilic parameter defined by equation 1.6 [134] where P_X is the octanol/water partition coefficient of the derivative and P_H of the parent compound:

$$\pi_X = \log P_X - \log P_H \qquad (1.6)$$

N,N-bis(2-chloroethyl) mustard group is a very lipophylic moiety (π value of 1.0 [134]) and the resulting simple phenyl derivatives are very insoluble in water: the octanol/water partition coefficient (log P) for *N,N*-bis(2-chloroethyl) aniline **(5)** is 2.9.[134]

The work of Bardos *et al.* [88,122] showed that the maximum efficacy and toxicity for the 4-substituted aniline mustards towards the solid tumour is found with rather lipophylic compounds whose log P is ca. 2.0 (equation 1.7 and 1.8).

$$\log (1/C\,ED_{90}) = -1.19\,\sigma^{-} + 0.75\,I_{Br} - 1.00\,\pi - 0.53\,\pi^2 + 3.84 \qquad (1.7)$$
$$n = 14,\ r = 0.94$$

$$\log (1/C\,LD_{50}) = -1.31\,\sigma^{-} + 0.51\,I_{Br} - 0.69\,\pi - 0.35\,\pi^2 + 3.87 \qquad (1.8)$$
$$n = 18,\ r = 0.93$$

Similar QSAR (equations 1.9 and 1.10) have been formulated by Hansch and his co-workers using antileukemia activity data from the National Cancer Institute for other aniline mustards [type **(4)** with X=Cl].[119,120] In these equations, C_{125} = molar dose (mol kg-1) producing a 25% life-span increase (T/C=125) against the L1210 leukemia in mice whereas C_{180} = molar dose to elicit (T/C =180) against the P338 leukemia in mice. The indicator variable I_O=1 when *ortho* substituents are present on the aromatic ring (other factors being equal, *ortho* substitution produces more active congeners). The addition of a π^2 term in equations 1.9 and 1.10 did not improve the correlation. The negative coefficient for π in these equations implies that hydrophilic derivatives with P < 0 are more active against non-solid leukemia tumours. This is a contrast to the results obtained by Bardos and his collegues against the solid Walker tumour.

$$\log (1/C_{125}) = -0.96\,\sigma^{-} - 0.31\,\pi + 0.86\,I_O + 4.07 \qquad (1.9)$$
$$n = 19,\ r = 0.93$$

$$\log (1/C_{180}) = -1.39\,\sigma - 0.34\,\pi + 0.30\,I_O + 4.13 \qquad (1.10)$$
$$n = 16,\ r = 0.91$$

Ross [41,135] was the first to examine the reactivity of congeners of **(4)** (where X=Cl or Br) towards nucleophilic replacement by measuring their "percent hydrolysis" under standard conditions [30 min reaction in aqueous acetone 50%, v/v at 66°C]. Subsequently, Panthananickal *et al.* [119] showed that Ross' data correlated with equation 1.11. Further,

$$\log\ \%\ hyd = -1.42\,\sigma + 0.45\,I_O + 0.70\,I_{Br} + 1.21 \qquad (1.11)$$
$$n = 42,\ r = 0.95$$

the data obtained by Bardos *et al.* [88] for the rate of alkylation by NBP of 4-substituted congeners of **(4)** (where X=Cl, Br or I) is correlated by equation 1.12, where I=1 for X=Br or I and I=0 for X=Cl (i.e. the Cl derivatives are about ten times less reactive).

$$\log k = -1.92\,\sigma^{-} + 1.12\,I - 1.77 \qquad (1.12)$$
$$n = 14,\ r = 0.97$$

Equations 1.11 and 1.12 show that two indexes of chemical reactivity (% hydrolysis and the rate of NPB alkylation) show good parallelism. The large negative value of ρ for the σ term is consistent with an aziridinium ion intermediate (*Scheme 1.12*), highly dependent on the electron density on nitrogen.[136] The positive value (+ 0.45) in equation 1.11 for the indicator variable I_o (I_o = 1 for the ortho substitution) shows that ortho substituents increase the rate of hydrolysis by a factor of ca. 3, no doubt by twisting nitrogen out of conjugation with the π electrons of the aromatic ring, thus making it more nucleophilic. The coefficient of variable I_{Br} in equation. 1.11 shows that 2-bromoethyl substituents hydrolyse ca. 5-fold more rapidly than Cl substituents. Other data show that branching at the β carbon of the mustard "arm" produces a drastic increase in reactivity.[17]

The coefficients of both the indicator variables (I_0, I_{Br}) and the σ parameter in equations 1.11 and 1.12 are in reasonable agreement with those of equations 1.7 to 1.10. This suggests that aziridinium ion intermediate formation is a common, critical factor for both the hydrolysis and the anticancer action of aniline mustards which justifies the use of Ross' hydrolysis model for predicting the effect of substituents on *in vivo* cytotoxic activity. However, although the toxicity of aryl mustards also relates to their ability to alkylate cellular DNA, additional factors such as drug transport and metabolism as well as DNA repair have also to be considered *in vivo* .

The reactivity of aromatic mustards is dependent on both the electronic properties of the aromatic substituent and the nucleofugacity (leaving ability) of the X substituent. This latter property can be measured by the leaving-group constant *L*, defined as the S_N2 rate ratio for nucleophilic displacement of X relative to Br (for which *L*=0) and *b* (a constant characteristic of the nucleophile)[137, 138] as expressed by equation 1.13,

$$\log (k_X/k_{Br}) = b\,L \qquad (1.13)$$

Palmer *et al.* [64] studied nucleofugacity effects for a series of aniline mustard congeners of **(4)** [where X were the four most commonly used leaving groups: Cl, Br, I and OSO_2CH_3 (OMs), and Y=H or NO_2] in relation to biological data [ED_{50} values - the concentration of drug necessary to reduce cell numbers to 50% of controls] for two cell lines (UV4 and AA8). The biological activity data, however, did not parallel chemical reactivity. For unsubstituted aniline mustards (Y=H), the OMs compound (with the best leaving group, *L*= -2.05) was the least toxic and the Cl compound (*L*= -1.61) was the least toxic of the halogen derivatives. This agreed with earlier data for *in vivo* potency where bromo mustards were found to be on average 3-fold more potent than the corresponding chloro mustards.[119] The 4-nitroaniline mustards ($Y=NO_2$) were generally much less toxic

(20-200 fold) than unsubstituted aniline mustards (Y=H), with the exception of the OMs compounds, where the difference was only 2-fold. Since aniline mustards have low water solubility, hydrophilic groups would be desirable for antileukemia drugs, with the OMs group (π= - 0.88) being the most favoured, followed by Cl (π= - 0.71) (see ***Table 2.1*** for some π and L values). ADEPT requires aniline mustard substrates that are highly sensitive to electronic substituent effects (in order to get the largest differential toxicity between prodrug and drug) and therefore a leaving group X that is sensitive to electronic effects of the aryl substituent. The OMs leaving group is insensitive to the electronic character of the aryl ring substituents. Further, although OMs is the most hydrophilic of the usual leaving groups available, it seemed to have little effect on the aqueous solubility of aryl mustards with Y=H or Y=NO_2. Thus, although the Cl leaving group gives a less potent aniline mustard, the superior solubility and sensivity to electronic effects of these compounds suggests their suitability for ADEPT testing.

Chapter 2

Results and Discussion 1:

Synthesis and Hydrolysis of Aromatic Nitrogen Mustards

2.1 Leaving group effects on the stability and reactivity of aniline mustards

As noted in Chapter 1, little systematic information is available about the stability and reactivity of aniline mustards bearing different leaving groups in the *N*-ethyl substituents. To address these questions, a series of simple (unsubstituted) *N,N*-bis-X-ethyl aniline mustards (X=Cl **(5)**, OH **(37)**, OSO_2CH_3 **(38)**, $OC(O)CH_3$ **(39)**, SCN **(40)**, ONO_2 **(41)**) were synthesised and their rates of decomposition briefly determined in aqueous DMSO at pH 7 - 9 and 37°C. The ionic strength was not maintained at a constant value.

2.1.1 Synthesis of aniline mustards (5) and (37) - (41)

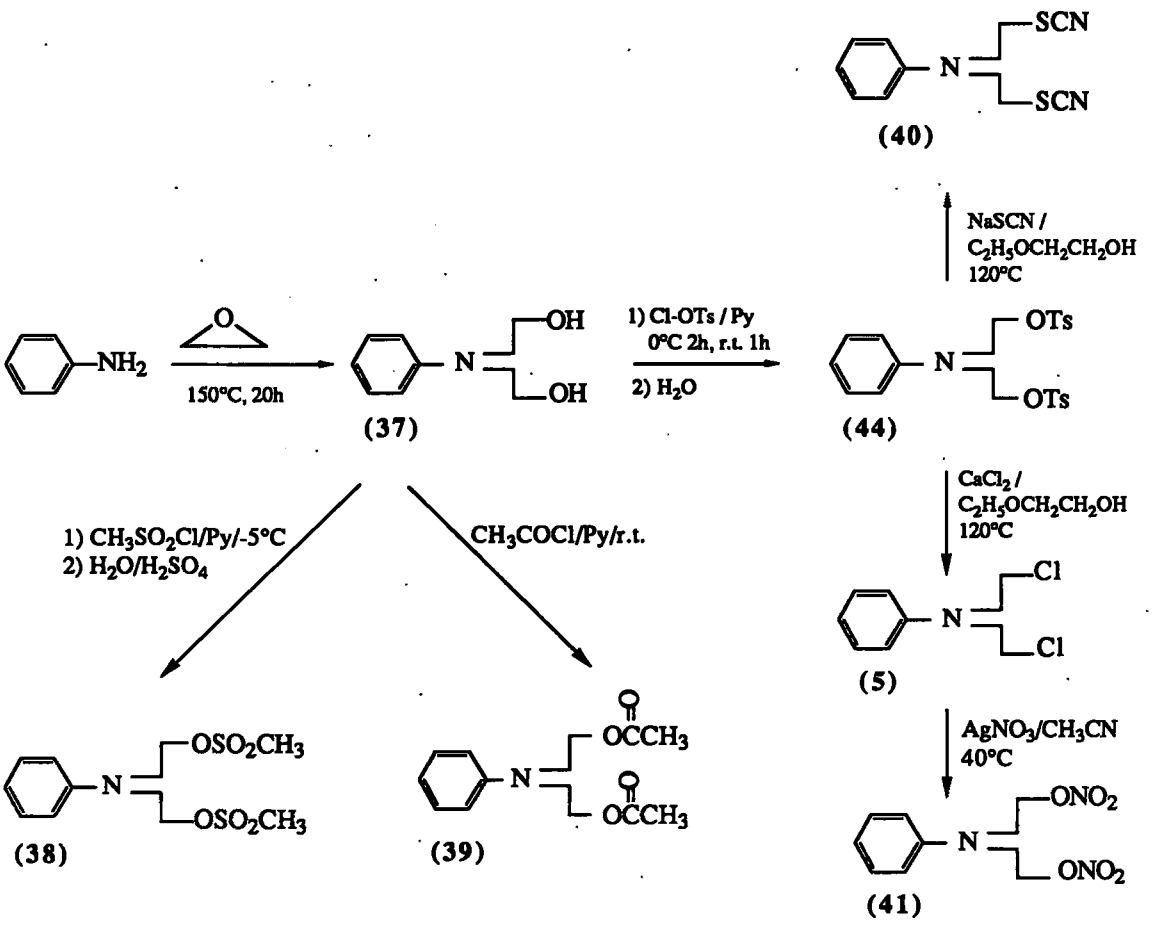
Synthesis of all these aniline mustards i.e., (bis-2-chloroethyl) **(5)**, (bis-2-hydroxyethyl) **(37)**, (bis-2-methanesulphonyloxyethyl) **(38)**, (bis-2-acetoxyethyl) **(39)**, (bis-2-thiocyano-ethyl) **(40)** but not (bis-2-nitratoethyl) **(41)**, are described in the literature. Their syntheses which have several common steps, are conveniently summarised by *Scheme 2.1.*

The preparation of nitrogen mustards usually proceeds via the corresponding bis(2-hydroxyethyl) amino derivative **(37)**. This was prepared by the method of Ross,[41,43] which involves reaction of the amine (in our case aniline) with ethylene oxide in a Carius tube at 150°C for 20h. At lower temperatures, large amounts of the monosubstituted product are produced.[41] Only two equivalents of ethylene oxide were used to minimise unproductive formation of polymers of the type **(42)**.

$$RN[(CH_2CH_2O)_nCH_2CH_2OH]_2 \quad (42)$$

Ross' method was preferred over the reaction of aniline with halohydrins in the presence of a base [43] (usually calcium carbonate) to neutralize acid coproducts, and over the route of Vingiello *et al.* [139] involving cleavage of a morpholine substituent to the mustard moeity using zinc chloride and conc. hydrochloric acid at 180°C.

Most of the hydroxyethyl and halogenoethyl compounds are light-sensitive and develop deep colours on exposure to air, especially in dilute solutions.



Scheme 2.1 Syntheses of aniline mustards (5) and (37) - (41)

N,N-Bis(2-hydroxyethyl)aniline **(37)** was obtained as an impure solid. It was recrystallised five times from benzene to give pure white plates in 71% yield. The melting point 55.4-56°C agreed well with the literature m.p. 55°C.[43] The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed two sets of triplets (8H) centred at 3.6 and 3.8 ppm, attributed to the hydroxyethyl CH_2 groups, a multiplet (5H) centred at 7.0 ppm for the aromatic protons and

an exchangeable broad singlet (2H) due to the two OH groups. The IR spectrum showed a strong absorption at 3350 cm^{-1} for the alcohol groups, and absorptions of medium intensity at 1600, 1500, 1330 and 750 cm^{-1} characteristic of a monosubstituted aromatic ring.

Synthesis of *N,N*-bis(2-methanesulphonyloxyethyl)aniline **(38)** and *N,N*-bis-(2-acetoxyethyl)aniline **(39)** involved reaction of the bis(2-hydroxyethyl) intermediate **(37)** with methanesulphonyl chloride in pyridine at -5°C over 30 min for **(38)**, and with acetyl chloride in pyridine at room temperature over 3h for **(39)**. This route was previously used by Cohen *et al.* [140] and Papanastassiou *et al.* [7] for the synthesis of **(38)**; the first synthesis of **(39)**, reported by Ross [141,142] involved reaction of **(37)** with hot Ac_2O and AcONa. Both compounds were obtained as impure oils in 56 and 69% yield, respectively. They were purified by silica column chromatography using DCM for **(38)** and ether/DCM (1:1,v/v) for **(39)**. The yield of pure **(38)** after chromatography was low (*ca.* 36%). It was much easier to convert **(38)** into the crystalline hydrochloride salt **(43)** by passing dry gaseous HCl through a solution of crude **(38)** in chloroform until saturated. The solution was then refrigerated and the precipitated **(43)** was filtered, washed with cold chloroform (without exposure to air), and recrystallised from cold acetone to give colourless crystals in 75% yield showing m.p. 77-82°C (lit.[140] m.p. 98-105°C). The ^{1}H-NMR spectra of **(38)** and **(43)** in $CDCl_3$ and $(CD_3)_2SO$, respectively, were similar. Namely, a singlet (6H) at 2.9 ppm for **(38)** and 3.2 ppm for **(43)** due to the two SO_2CH_3 methyl groups; two sets of triplets (4H) centred at 3.7 and 4.3 ppm with a J=6.8 Hz for **(38)** and 3.75 and 4.35 ppm with a J=6Hz for **(43)**, corresponding to the NCH_2 and OCH_2 groups, respectively; and a multiplet centred at 7.0 ppm for both compounds but integrating to 5H for **(38)** and to 6H for **(43)** for the aromatic protons, with an additional proton due to NH^+ for **(43)**. The IR spectra of both **(38)** and **(43)** showed characteristically strong absorptions at 1350 and 1175 cm^{-1} due to the OSO_2CH_3 group.[143] The MS(EI) spectrum for **(38)** showed a peak at m/z=337 due to M^+, while the MS(FAB^-) spectrum for **(43)** showed a peak at m/z=372 corresponding to $M\text{-}H^+$.

The ^{1}H-NMR spectrum in $(CD_3)_2CO$ of *N,N*-bis(2-acetoxyethyl)aniline **(39)** showed a singlet (6H) at 2.0 ppm, due to the methyl ester groups; a double triplet (4H each) centred at 3.7 and 4.3 ppm with J=7.5 Hz for the NCH_2 and OCH_2 groups, respectively; and a multiplet (5H) centred at 7.05 ppm for the aromatic protons. Its IR spectrum showed a strong absorption at 1670 cm^{-1} due to the ester carbonyl, as well as peaks at 1600,1500 (aromatic), and 1380 cm^{-1} (C-O, ester). The MS(FAB^+) spectrum of **(39)** had a strong peak at m/z=266 corresponding to MH^+.

The synthesis of *N,N*-bis(2-chloroethyl)aniline **(5)** previously described in the literature [41,43,140] involved direct replacement of the hydroxyl groups of **(37)** by halogen using either $POCl_3$, PCl_5, or occasionally $SOCl_2$. A slightly different route was used here, involving activation of the diol by conversion of **(37)** into the 4-toluenesulphonyl chloride derivative **(44)**,[47,144] followed by displacement of the tosyl ester group using calcium chloride in 2-ethoxyethanol.[145] This reaction took place at 120°C for 1.5h. After extraction into benzene, *N,N*-bis(2-chloroethyl)aniline **(5)** was obtained impure in 60% yield. It was recrystallised from toluene/n-hexane (1:1, v/v) giving a solid showing m.p. 43-45°C (lit.[43] m.p. 45°C). The MS(EI) spectrum showed m/z=217 corresponding to M^+ and peaks at m/z=183 and 168 for the liberation of a chloride atom (M^+ - Cl) or for (M^+ - CH_2Cl), respectively.

Synthesis of *N,N*-bis(2-ethyltoluene-4-sulphonate)aniline **(44)** followed a route similar to one described in the literature [140,146] involving reaction of **(37)** with toluene-4-sulphonylchloride in pyridine at 0°C for 2h and a further 1h at room temperature, followed by treatment with water to give impure **(44)** in 82% yield, and in 75% yield after recrystallisation from ethanol. The pure white needles showed m.p. 88-89°C (lit.[140] m.p. 90-91°C) and its MS(EI) spectrum showed m/z=489 corresponding to M^+. The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed a singlet (6H) at 2.4 ppm for the methyl protons of the tosyl group; a double triplet (8H) centred at 3.6 and 4.1 ppm attributed to the CH_2 groups; and two multiplets centred at 6.8 and 7.5 ppm integrating, respectively, to 5H and 8H (associated with the tosyl group) for the aromatic protons.

The synthesis of *N,N*-bis(2-thiocyanoethyl)aniline **(40)** involved reaction of the tosyl derivative **(44)** with sodium thiocyanate in 2-ethoxyethanol at 120°C for 3h, a different route from the one previously described in the literature. [147-150] The product was purified by silica column chromatography using DCM as eluent, and further recrystallised twice from DCM/n-hexane (1:1, v/v) to give white crystals in 49% yield, showing m.p. 68-70°C (lit. m.p. 73-74°C).[147,148] The MS(FAB$^+$) spectrum showed m/z=264 due to MH^+, and the ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed two triplets at 3.3 and 3.8 ppm, both with J=8 Hz and integrating to 4H, attributed to the NCH_2 and OCH_2 groups, respectively; and a multiplet (5H) centred at 7.0 ppm for the aromatic protons. The IR spectrum confirms the -SCN rather than -NCS structure because of a sharp band of medium intensity at 2153 cm^{-1} due to the C≡N moiety and a strong band at 722 cm^{-1} due to C-S moiety.

Finally, the synthesis of *N,N*-bis(2-nitratoethyl)aniline **(41)** involved reaction of the chloro derivative **(5)** with silver nitrate in acetonitrile at 40°C for 15h. This gave **(41)** as

an impure compound in 66% yield. It was purified by silica column chromatography using ether/n-hexane (1:2, v/v) as eluent, but the relatively low yield (41%) of recovered pure yellow oil reflected the difficulty of the purification. The MS(EI) spectrum showed m/z=271 corresponding to M^+. The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed two sets of triplets (4H each) centred at 3.8 and 4.75 ppm both with J=7Hz, attributed to the NCH_2 and OCH_2 groups, respectively; and a multiplet (5H) centred at 7.0 ppm for the aromatic protons.

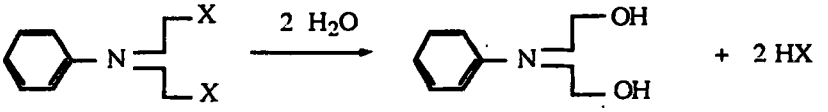
2.1.2 Hydrolysis of aniline mustards (5) and (38) - (41)

The reactions were complicated at the outset by the low aqueous solubilities of the aniline mustard substrates. Some indication of these difficulties is evident in Ross' early studies [43] which were carried out in aqueous acetone (1:1, v/v) at 66°C. The aim of the present work was to obtain data in buffered, aqueous media at 37°C, to facilitate assessment of their behaviour *in vivo*.

The low aqueous solubility of the aniline mustards was partially circumvented by using aqueous-DMSO. The ionic strength was not maintained at a constant value.

2.1.2.1 Autotitration assay

It was anticipated that the reactions could be followed by autotitration of the 2 moles of acid HX released on hydrolysis:



(2.1)

In practice, this method proved difficult because relatively high (*ca.* 10^{-4}M) substrate concentrations were required to obtain accurate autotitration. In turn, this required relative high concentrations of DMSO (up to 30%, v/v) to maintain homogeneous reaction solutions, with a concomitant decline in electrode performance. With DMSO > 5% v/v, the sensitivity of the calomel and glass electrodes noticeably decreases after *ca.* 5h at 37°C. At pH 7 and 37°C, hydrolysis of some of the aniline mustard substrates was quite slow, which led to problems additional to the deterioration of electrode sensitivity. Autotitration of the released acid with dilute (5mM) aqueous NaOH requires sparging with N_2 to avoid errors arising from CO_2 absorption. At 37°C, this caused significant evaporation of water from the

reaction solution and therefore further inaccuracies. For all of these reasons, examination of the hydrolysis of the aniline mustard substrates was limited to just a few experiments.

The reactions were carried out by injecting a stock solution of the aniline mustard in DMSO (100µl, $8x10^{-2}$M) into a solution of aqueous DMSO (either 99:1, 9:1 or 7:3, v/v, 25cm^3) at pH 7.0 or 9.0, contained in the thermostatted reaction vessel (37°C) of the autotitrator. The final concentration of substrate was ca. $3.2x10^{-4}$ M. The solution was stirred vigorously under N_2 and the pH was maintained at the preset value by the autotitrator by the addition of standard aqueous NaOH (5mM). The autotitrator recorded the volume of NaOH with time. The reactions were monitored until the addition of NaOH ceased, or for *ca.* 24h. Attempts to evaluate the *pseudo* first order rate coefficient k_O (Equation 2.2) either from the

$$\text{Rate} = k_O\,[\text{aniline mustard}] \qquad (2.2)$$

initial rate of addition of NaOH or from plots of log $(V_\infty - V_t)$ *versus* time, where V_t and V_∞ = volume of 5mM NaOH added by the autotitrator, at time t and infinity, respectively, were unsatisfactory: the plots were poorly linear probably because of electrode deterioration at long reaction times and infinity values were difficult to evaluate. Thus, k_O values were calculated from reaction half-lives [$t_{1/2}$= time for addition of 50% of the theoretical amount of 5mM NaOH] estimated from the autotitrator plots, and they represent an average of the individual rate coefficient for expulsion of the first and second *N*-ethyl-2-substituents. *Pseudo* first order rate coefficients (k_O) and half-lives ($t_{1/2}$) for the hydrolyses of **(5)**, **(38)**, **(39)**, **(40)** and **(41)** under various conditions are summarised in ***Table 2.1***. Also given are pKa values for HX which is relate to the nucleofugacity of X in protic solvents and Hansch lipophilicity constants for X^-. From the data for **(5)**, the hydrolysis rate is not strongly dependent on the % DMSO. From the data for **(5)** and **(38)**, the hydrolysis rate is not strongly dependent on pH, and the reactions do not appear to be base-catalysed.

The low quality of the experimental data prevented analysis by the time ratio method to obtain individual values of k_1 and k_2. It is evident from the ion exchange chromatography (IEC) results, however, that k_1 and k_2 are of similar magnitude (see ***Table 2.5***). Thus, the average value (k_O) reported in ***Table 2.1*** is a good measure of the relative reactivity of the various mustards and the effect of nucleofugacity on reactivity.

Table 2.1

Hydrolysis rates for mustards (5) and (38) - (41) in aqueous DMSO at 37°C obtained by autotitration of the acid released

Mustard	X	pH	H_2O/ DMSO (v/v)	k_o (s^{-1})	$t_{1/2}$	pK_a of HX^{α}	π^{β}	L^{γ}
(5)	Cl	7	99:1	$2.1x10^{-4}$	56 min	-6.1	0.71	-1.61
		9	99:1	$2.1x10^{-4}$	56 min			
		9	9:1	$1.2x10^{-4}$	96 min			
		9	7:3	$1.0x10^{-4}$	114 min			
(38)	OSO_2CH_3	7	99:1	$3.3x10^{-3}$	3.5 min	-1.2	-0.88	- 2.05
		9	99:1	$3.9x10^{-3}$	3.0 min			
(39)	$OC(O)CH_3$	7	99:1	no reaction over 24h	-	+4.76	0.64	- 4.68
		9	99:1	$1.2x10^{-5}$	16h			
(40)	SCN	7	7:3	no reaction over 24h	-	-1.85	-0.48	?
		9	7:3	no reaction over 24h	-			
(41)	ONO_2	9	9:1	$2.2x10^{-6}$	89h	-1.44	-1.4	-1.90
		9	7:3	$1.9x10^{-6}$	102h			

(α) in water, 25°C [151]

(β) π Hansch lipophilicity constant, according to eqn. 1.6 [134]

(γ) L Leaving group constant, according to eqn. 1.13 [138]

2.1.2.2 HPLC assay for aniline mustards (40) and (41)

According to the autotitration experiments, aniline mustard **(40)** appears to be stable to hydrolysis at pH 7.0 and 9.0 at 37°C: no measurable consumption of 5mM NaOH was apparent over 72h.

For aniline mustard **(41)**, slow consumption of 5mM NaOH was apparent. It was of

interest, however, to identify the products and to confirm that the slow consumption of base does not relate to reaction with atmospheric CO_2.

Accordingly, the reaction solutions for aniline mustards **(40)** and **(41)** were also analysed by HPLC against authentic *N,N*-bis(2-hydroxyethyl)aniline **(37)**, 4-phenyl-morpholine **(45)**, and the substrates, themselves.

2.1.2.2.1 Aniline mustard (40)

An aliquot of the reaction solution for the hydrolysis of *N,N*-bis(2-thiocyanoethyl)aniline **(40)** in aqueous DMSO (7:3, v/v) at pH 9.0 and 37°C, followed by autotitration of acid released (Section 2.1.2.1) for 72h, was analysed by HPLC. At this time there had been no significant consumption of 5mM NaOH and apparently no reaction.

The HPLC chromatogram showed 4 peaks whose relative peak areas and retention times (Rf) are summarised in ***Table*** **2.2**. By comparison with authentic compounds, the peak at Rf=2.73 min is the solvent DMSO, the peak at Rf=3.63 min is *N,N*-bis(2-hydroxyethyl)-aniline **(37)**, the peak at Rf=14.79 min is unreacted substrate, and the peak at Rf=22.89 min is probably 5-phenyl-tetrahydro-1,2,5-dithiazepine **(46)**, the main product found by Dubinskaite *et al.* [147,148] for the decomposition of **(40)** in alkali, presumably formed by an intramolecular reaction. Significantly, 4-phenylmorpholine **(45)** (Rf=6.76 min) was not formed as a reaction product.

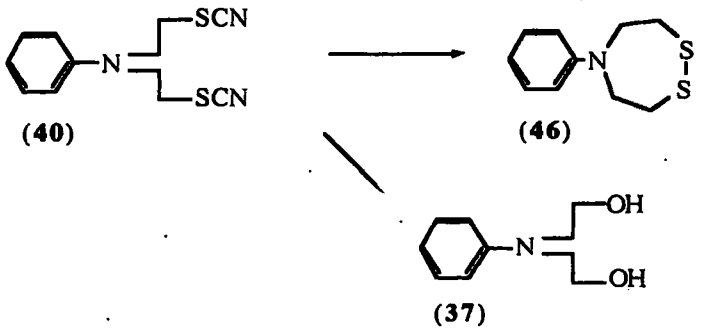
Table **2.2**

HPLC assay of hydrolysis products for aniline mustard **(40)** *in aqueous DMSO (7:3,v/v) at pH 9 and 37°C after 72h reaction:* λ*=263nm,* $[\mathbf{40}]_{init}$ *ca.* 3.2×10^{-4}*M*

Compound	Retention time (Rf) (min)	Peak area (%)
DMSO	2.73	10
N,N-Bis(2-hydroxyethyl)aniline **(37)**	3.63	10
N,N-Bis(2-thiocyanatoethyl)aniline **(40)**	14.79	65
5-Phenyl-tetrahydro-1,2,5-dithiazepine **(46)**	22.89	18

It is not possible from the data in ***Table*** **2.2** to determine the extent of reaction. Although substantial starting material **(40)** remains after 72h, the HPLC assay shows some decomposition in contrast to the autotitration results. The products formed are summarised

in *Scheme* 2.2.



Scheme 2.2 *Hydrolysis pathways for aniline mustard* (**40**) *in aqueous DMSO (7:3, v/v) at pH 9 and 37°C*

2.1.2.2.2 Aniline mustard (41)

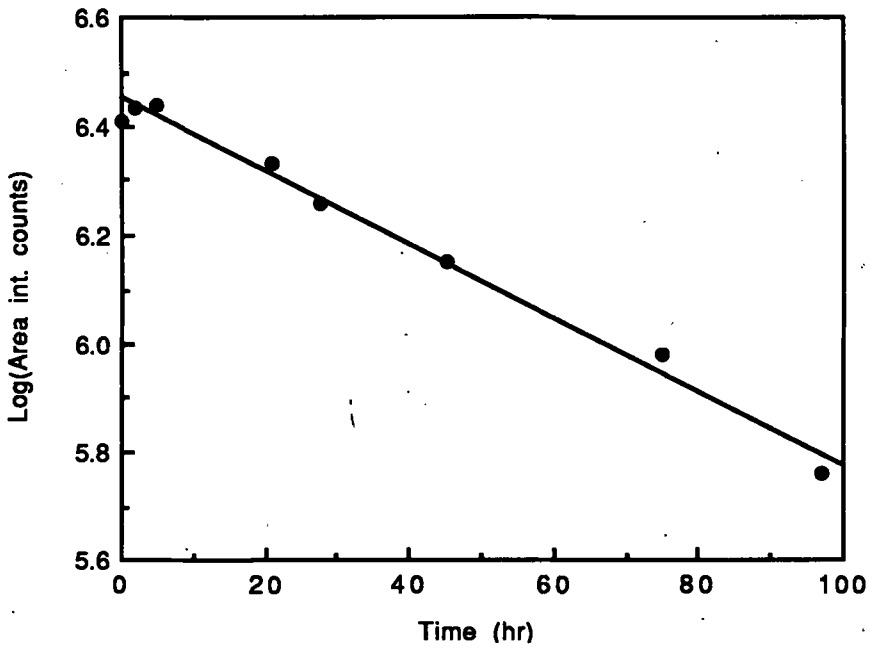
The hydrolysis of *N,N*-bis(2-nitratoethyl)aniline (**41**) in aqueous DMSO (7:3,v/v) at pH 9.0 and 37°C was assayed at timed intervals by HPLC as described in the Experimental (Section 5.4.2.1). The retention times (Rf) of peaks in the chromatogram, and their identification against authentic compounds, are summarised in *Table* 2.3. The variation of integrated peak areas with time are shown in *Figure* 2.1 and the slope of log (peak area) of (**41**) *versus* time gives $k_O = 1.9 \times 10^{-6}\ s^{-1}$. The peak at Rf=2.45 min with a constant area at all reaction times

Table 2.3

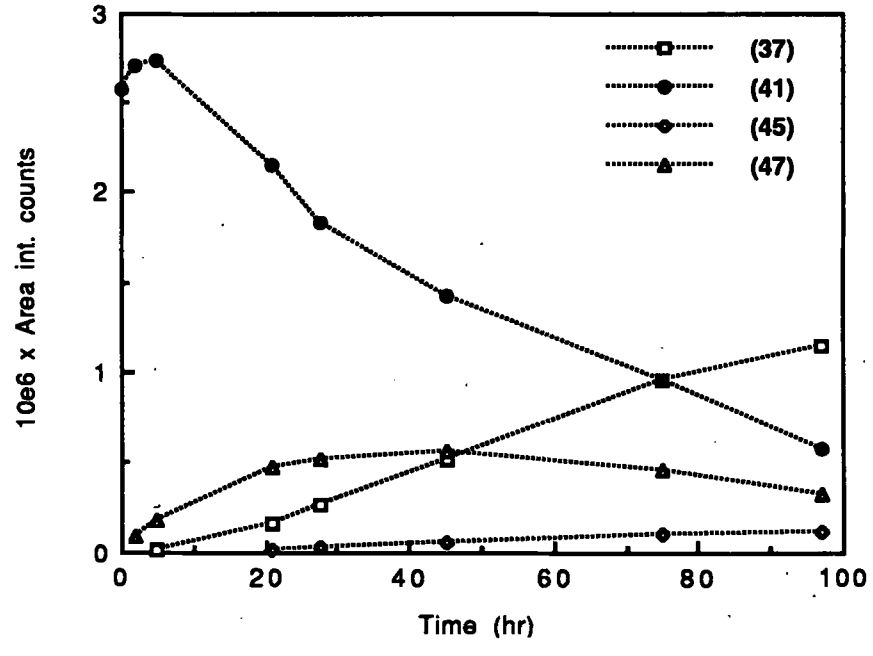
HPLC assay of hydrolysis products for aniline mustard (**41**) *in aqueous DMSO (7:3, v/v) at pH 9 and 37°C:* $[41]_{init}$ *ca.* 3.2×10^{-4}*M;* λ*=263 nm*

Compound		Retention time (Rf) (min)
DMSO		2.45
N,N-Bis(2-hydroxyethyl)aniline	(37)	3.78
N,N-Bis(2-nitratoethyl)aniline	(41)	10.65
4-Phenylmorpholine	(45)	7.21
Half-mustard	(47)	7.65

corresponds to the solvent DMSO; the peak at Rf=3.78 min showing a steady increase in area with time is the diol (**37**); the peak at Rf=7.65 min passing through a maximum area at *ca.* 40h was not identified against an authentic compound, but this behaviour is that expected



a) Disappearance of starting material **(41)**, Rf=10.65 min

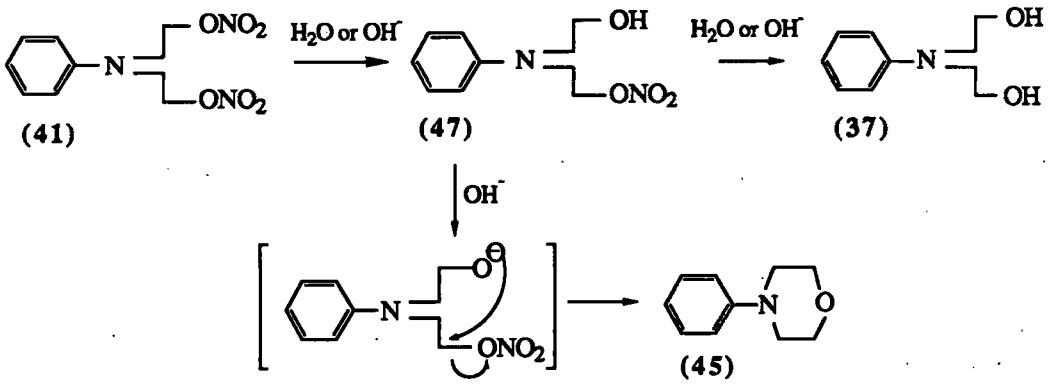


b) Disappearance of of starting material **(41)**, Rf=10.65 min and formation of products: **(37)**, Rf=3.78 min; **(45)**, Rf=7.21 min; **(47)**, Rf=7.65 min

Figure 2.1 Hydrolysis of aniline mustard (41) in aqueous DMSO (7:3, v/v) at pH 9 and 37°C over 97h: $[41]_{init}$ ca. $3.2x10^{-4}M$; λ=263 nm

for the half-hydrolysed mustard [*N*-(2-hydroxyethyl)-*N*-(2-nitratoethyl)aniline] **(47)** in *Scheme* 2.3; the small peak at Rf=7.21 min, which appears after 20h, corresponds to phenylmorpholine **(45)**; and finally, the peak at Rf=10.65 min showing a steady decrease in area with time, is the starting substrate **(41)**.

The HPLC assay shows that the hydrolysis of **(41)** in aqueous DMSO (7:3, v/v) at pH 9.0 and 37°C is slow (k_O= 1.9x10^{-6} s^{-1}), in good agreement with the autotitration results (1.9x10^{-6} s^{-1}). The decomposition pathways are outlined in *Scheme* 2.3.



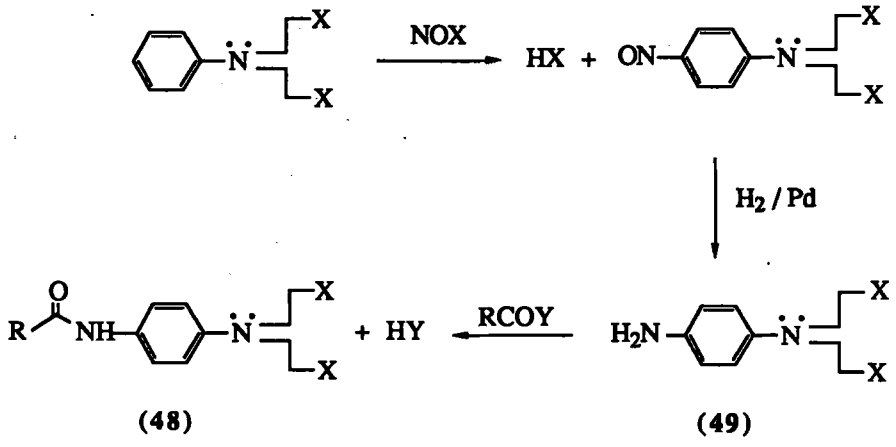
Scheme 2.3 Hydrolysis pathways for aniline mustard (41) in aqueous DMSO (7:3,v/v) at pH 9 and 37°C

2.2 Synthesis of potential aniline mustard prodrug/drug combinations

The chemotherapeutic properties of aniline mustards are well documented as outlined in Chapter 1. It is clear that the most potent compounds bear electron-donating substituents in the aromatic ring and good leaving groups (eg. halide, alkyl sulphonate) in the bis-*N*,*N*-dialkyl substituents. Other work at the PHLS Porton Down Laboratory and the Charing Cross Hospital, indicated that arylamidases might be suitable enzymes for ADEPT procedures. These considerations pointed to the potential utility of 4-(*N*-acylamino)aniline mustards **(48)** as prodrugs for ADEPT procedures.

Chart 4 Structures (48) and (49)

This section reports the synthesis of the potential prodrug/drug combinations (48) /(49), with R=CH_3 or CF_3 and X=Cl, OSO_2CH_3 or $OC(O)CH_3$, for subsequent chemical and biochemical evaluation. Most of these syntheses followed a common pathway (*Scheme* 2.4) involving introduction of a 4-amino substituent into the appropriate aniline mustard, via acid-catalysed nitrosation followed by catalytic reduction and finally acetylation of the free amino group.

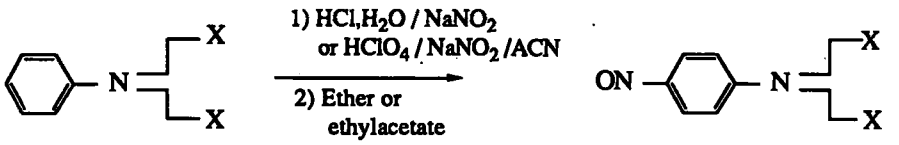


Scheme 2.4 Common pathway for the synthesis of 4-(N'-acylamino) aniline mustards

2.2.1 Synthesis of 4-nitroso aniline mustards (50) - (53)

These compounds were obtained by a common, published[41] method involving nitrosation of the parent aniline mustard in acidic aqueous sodium nitrite at 0 to -5°C according to *Scheme* 2.5. The aniline mustard was dissolved in aqueous HCl or $HClO_4$ (if X≠Cl, to avoid displacement of X by Cl) plus some acetonitrile when necessary to solubilise the mustard. The solution was cooled to 0 to -5°C and aqueous $NaNO_2$ (*ca.* 1.1 eq) was then added dropwise over 30 min, with vigorous stirring. The mixture was stirred for a further 30 min at 0 to -5°C. For compounds (50) and (51), the reaction solution was neutralized before extraction of the green, 4-nitroso product into an organic solvent. This method was first used by Ross *et al.* [41] for the synthesis of (52) and subsequently for (50)[152] and (51),[7] but compound (53) doesn't seem to have been prepared previously.

N,N-Bis(2-hydroxyethyl)-4-nitroso-aniline (50) was obtained by nitrosation of *N,N*-bis(2-hydroxyethyl)aniline (37) in aqueous HCl, followed by neutralization with saturated $NaHCO_3$, saturation of the solution with sodium perchlorate and extraction into



(37), (50) : X=OH

(38), (51) : $X=OSO_2CH_3$

(5), (52) : X=Cl

(41), (53) : $X=ONO_2$

Scheme 2.5 Synthesis of 4-nitroso mustards (50) - (53)

ethyl acetate. Pure **(50)** in 74% yield was obtained by silica column chromatography using ethyl acetate as eluent. This showed m.p. 134-136°C (lit. m.p. 137-138°C)[152] and m/z=210 corresponding to M^+ in the MS(EI) spectrum. The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed two singlets (4H each) at 3.0 and 3.8 ppm for NCH_2 and CH_2OH groups, respectively; a broad overlapping singlet (2H) at 3.8 ppm for the two OH groups; and an AB quartet (4H) at 6.9-7.7 ppm with J=10.1 Hz, for the aromatic protons. The most significant feature of the IR spectrum was a strong absorption at 3350 cm^{-1} due to the alcohol groups.

N,N-Bis(2-methanesulphonyloxyethyl)-4-nitroso-aniline **(51)** was obtained similarly by nitrosation of *N,N*-bis(methanesulphonyloxyethyl)aniline hydrochloride **(43)** in aqueous acetonitrile containing $HClO_4$. After neutralization with NaOH to pH 7, extraction into DCM gave impure **(51)** which was purified by silica column chromatography using ethyl acetate as eluent. The pure product was obtained in 62% yield showing mp. 116-118°C (lit.[7] mp. 117-118.5°C), and m/z=367 in the MS(FAB^+) spectrum corresponding to MH^+. The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed a singlet (6H) at 3.1 ppm for the methyl protons; two sets of triplets (4H each) centred at 4.1 and 4.6 ppm with a J=6 Hz, for the NCH_2 and OCH_2 groups, respectively; and an AB quartet (4H) at 7.1-7.8 ppm with J=10.3 Hz for the aromatic protons. The IR spectrum showed as most relevant feactures, bands at 1350 and 1175 cm^{-1} characteristic of the OSO_2CH_3 group.

N,N-Bis(2-chloroethyl)-4-nitroso-aniline **(52)** was obtained by nitrosation of *N,N*-bis(2-chloroethyl)aniline **(5)** in aqueous HCl followed by extraction into ether. After recrystallisation from ether, pure **(52)** was obtained in 79% yield with m.p. 79-80°C (lit. m.p.

79-80°C) [41] and m/z=246 in the MS(EI) spectrum corresponding to M^+. The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed a double triplet centred at 4.0 ppm (integrating to 8H) for all the CH_2 protons; and an AB quartet (4H) at 6.9-7.9 ppm with J=9.0 Hz for the aromatic protons. The IR spectrum showed bands at 3268 (NH) 1600, 1500 (Ar) and 1380 cm^{-1} due to the nitroso group.

N,N-Bis(2-nitratoethyl)-4-nitroso-aniline **(53)** was obtained by nitrosation of *N,N*-bis(2-nitratoethyl)aniline **(41)** in aqueous acetonitrile (1:5, v/v) using $HClO_4$ and $NaNO_2$, followed by extraction into ether giving an impure product in 44% yield. After purification by silica column chromatography using DCM as eluent, pure **(53)** was obtained in 30% yield with m.p. 85-87°C and a satisfactory elemental analysis. The MS(EI) spectrum showed m/z=300 corresponding to M^+. The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed two sets of triplets (4H each) centred at 4.14 and 4.95 ppm with J=6.7 Hz, attributed to the NCH_2 and OCH_2 groups, respectively, and an AB quartet (4H) at 7.0-7.8 ppm with J=10.3 Hz for the aromatic protons. The IR spectrum shows a band at 1380 cm^{-1}, due to the nitroso group.

The assignment of the -N=O stretching frequency for aromatic nitroso compounds has been a matter of some difficulty. The IR spectra of all the 4-nitroso aniline mustards show a band at 1340-1380 cm^{-1} due to the nitroso group, which agrees better with Nakamoto and Runde[153] than the value of 1500 cm^{-1} suggested by Bellamy.[154,155]

2.2.2 Synthesis of 4-amino aniline mustards (10)/(54) and (55)/(56)

These compounds were obtained by catalytic hydrogenation of the appropriate 4-nitroso-aniline mustards in methanol using 15% palladium on charcoal as catalyst. This procedure was first reported by Ross [41] for the synthesis of **(10)**.

The 4-nitroso-aniline mustard substrate was stirred under hydrogen at atmospheric temperature and pressure until the calculated volume of hydrogen had been consumed and the green colour had disappeared. The only difficulties related to the high reactivity of the neutral 4-amino products, which quickly decomposed (polymerised or oxidised). Thus, the products were converted immediately to either the more stable N-acyl prodrugs or the less reactive hydrochloride or sulphonate salts [by treatment of the reaction solution (after filtration of the catalyst) with either methanolic HCl or methanolic methane sulphonic acid]. Both *N,N*-bis(2-chloroethyl)-4-amino-aniline **(10)** and *N,N*-bis(2-methane-sulphonyloxyethyl)-4-amino-aniline **(55)** were not isolated as neutral compounds but as their hydrochloride **(54)** or methanesulfonate **(56)** salts, respectively.

Impure *N,N*-bis(2-chloroethyl)-4-amino-aniline hydrochloride salt **(54)** was obtained as a brown residue in 81% yield. A small amount was twice recrystallised from methanol/ether (1:1, v/v) to obtain a pure product as almost colourless plates with m.p. 245-258°C [lit.[156] m.p. 250-260°C (decomp.)], and a ^{1}H-NMR spectrum in $(CD_3)_2SO$ showing a singlet (8H) at 3.8 ppm for the CH_2 groups; an AB quartet (4H) at 6.8-7.3 ppm with a J=9.4 Hz for the aromatic protons; and a broad singlet at 10.3 ppm for the NH proton.

Impure *N,N*-bis(2-methanesulphonyloxyethyl)-4-amino-aniline methanesulfonate salt **(56)** was obtained in *ca.* 40% yield as oily residue. It was difficult to recrystallise and therefore used immediately in the next stage of the synthesis. Synthesis of **(56)** has not been reported.

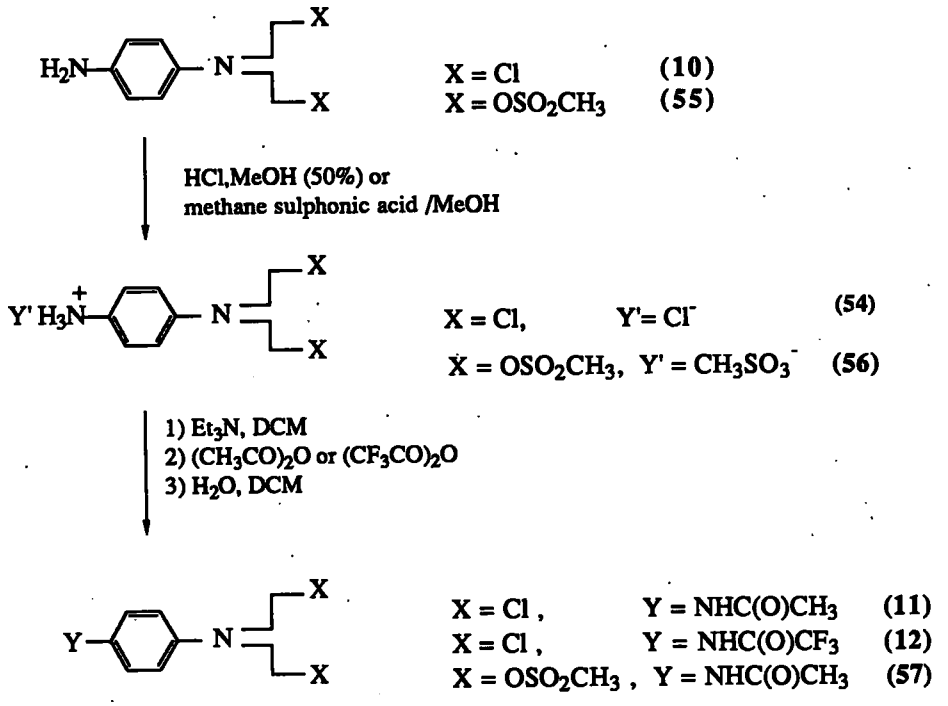
2.2.3 Synthesis of potential aniline mustard prodrugs (11),(12), (57) and (58)

N-[4-[*N'*,*N'*-Bis(2-chloroethyl)amino]phenyl]acetamide **(11)**, *N*-[4-[*N'*,*N'*-bis(2-chloro ethyl)amino]phenyl]trifluoroacetamide **(12)** and *N*-[4-[*N'*,*N'*-bis[2-methanesulphonyloxy ethyl]amino]phenyl]acetamide **(57)** were all obtained by acetylation of the appropriate 4-amino substrate, immediately following the above reduction (*Scheme 2.6*). Following isolation, the crude 4-amino salt was treated with excess triethylamine plus acetic or trifluoroacetic anhydride. The yield of the reaction was usually low (*ca.* 20%) probably because the neutral 4-amino aniline mustard is very unstable. The literature suggests that for 4-amino-*N,N*-dialkylanilines, loss of the $N(CH_2CH_2X)_2$ moiety is very facile.[157,158]

The synthesis of both **(11)** and **(12)** was first reported by Ross *et al.*[40,41] Compound **(57)** is new but the 3-*N*-acetylamino analogue was synthesised by Papanastassiou[143] by a different approach involving esterification with methanesulfonyl-choride of 3-[*N,N*-bis-(2-hydroxyethyl)amino]acetanilide.

N-[4-[*N'*,*N'*-bis(2-chloroethyl)amino]phenyl]acetamide **(11)** was obtained in 25% yield after recrystallisation from ether/n-hexane (1:1, v/v), further purification by silica column chromatography using ether/DCM (3:1,v/v) as eluent and two further recrystallisations from ether/n-hexane (1:1, v/v). It gave m.p. 120°C (lit. [41] m.p. 124-126°C), m/z=274 corresponding to M^+ in the MS(EI) spectrum and a satisfactory elemental analysis. The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed two singlets, one at 2.1 ppm (3H) for the methyl protons, the other at 3.7 ppm (8H) for the protons in the CH_2 groups, and an AB quartet (4H)

at 6.7-7.5 ppm with a J=10 Hz for the aromatic protons. The IR spectrum showed a strong absorption at 1650 cm^{-1} for the amide carbonyl group.



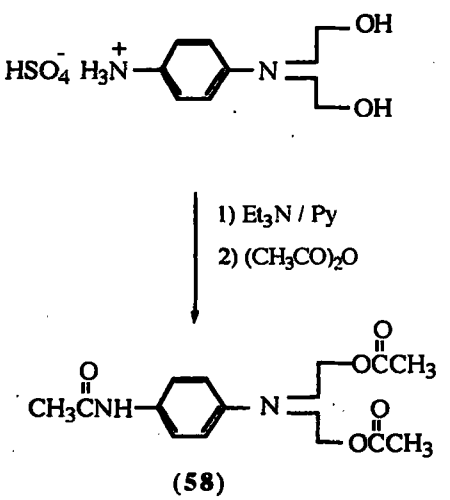
Scheme 2.6 Synthesis of the N-acyl potential prodrugs (11), (12) and (57)

Pure *N*-[4-[*N'*,*N'*-bis(2-chloroethyl)amino]phenyl]trifluoroacetamide **(12)** was obtained in 20% yield after a series of purification steps involving recrystallisation from ether/n-hexane (1:1,v/v), silica column chromatography using ether/n-hexane (1:1,v/v) and further recrystallisation from ether/n-hexane (1:1,v/v). The shiny silver crystals showed mp. 101-103°C (lit.[40] m.p. 109-110°C), m/z=328 corresponding to M^+ in the MS(EI) spectrum and a satisfactory elemental analysis. The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed a singlet (8H) at 3.8 ppm for the CH_2 groups; an AB quartet (4H) at 6.8 - 7.6 ppm with J=10.5 Hz for the aromatic protons; and a broad exchangeable singlet (1H) at 10.1 ppm for the NH proton. The IR spectrum showed a strong absorption at 1700 cm^{-1} for the trifluoroacetamide carbonyl group.

N-[4-[*N'*,*N'*-Bis[2-methanesulphonyloxyethyl]amino]phenyl]acetamide **(57)** was obtained as an impure syrup in 54% yield after silica column chromatography using ethyl acetate as eluent. It proved impossible to recrystallise. Normal phase HPLC using a silica column and several eluent mixtures (EtOAc/ACN/MeOH 98:1:1,v/v/v or EtOAc/DCM 9:1,v/v) also gave no purification. The MS(EI) spectrum showed m/z=394 corresponding to

M^+, and the ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed two singlets at 2.0 (3H) and 3.0 ppm (6H) for the methyl amide and OSO_2CH_3 groups, respectively; two sets of triplets (4H) centred at 3.8 and 4.4 ppm with J=6.3 Hz, for the NCH_2 and OCH_2 groups, respectively; an AB quartet (4H) at 6.8-7.5 ppm with a J=10.4 Hz for the aromatic protons; and a broad exchangeable singlet (1H) at 9.0 ppm for the NH proton. The IR spectrum showed a strong absorption at 1670 cm^{-1} due to the amide carbonyl and absorptions at 1350 and 1175 cm^{-1} for the mesyl OSO_2CH_3 groups.

Finally, *N*-[4-[*N'*,*N'*-bis(2-acetyloxy)ethyl]amino]phenyl]acetamide **(58)** was obtained by acetylation of commercially available *N*,*N*-bis(2-hydroxyethyl)-4-phenylene diamine sulphate with acetic anhydride in pyridine (***Scheme 2.7***), following prior conversion of the phenylene diamine salt into the free base by treatment with triethylamine for 10 min at room temperature. The acetylation was carried out at room temperature for 24h. After vacuum evaporation of the pyridine, the product was extracted into DCM. Compound **(58)** is not described in the literature and it was obtained in 31% yield after purification by silica column chromatography using ethyl acetate as eluent followed by two recrystallisations from DCM/n-hexane (1:1,v/v). The shining white crystals of **(58)** showed m.p. 64-66°C and gave a satisfactory elemental analysis (see Experimental). The MS(EI) spectrum showed m/z=322 corresponding to M^+ and the ^{1}H-NMR spectrum $(CD_3)_2CO$ showed two singlets at 1.9 (6H) and 2.0 ppm (3H) for the ester and the amide methyl groups, respectively; two sets of triplets (4H each) centred at 3.6 and 4.2 ppm with J=6.7 Hz for the CH_2 groups; an AB quartet (4H) at 6.8-7.5 ppm with J=10.2 Hz, for the aromatic protons; and a broad exchangeable singlet (1H) at 8.9 ppm for the NH proton. The IR spectrum showed a strong absorption at 1745 and 1670 cm^{-1} characteristic for ester and amide carbonyl groups, respectively.



(58)

Scheme 2.7 *Synthesis of the N-acyl potential prodrug (58)*

2.3 Stabilities of aryl mustards (5) and (10), (11) and (12) in aqueous phosphate buffer 3% (v/v) DMSO at pH 7.4 and 37°C

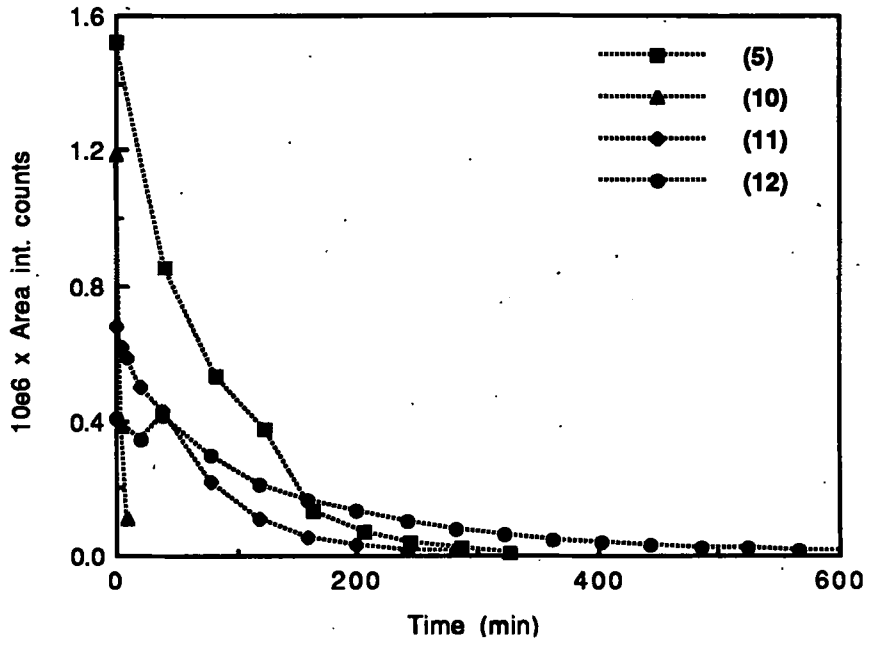
To progress the evaluation of aryl mustards *N*-[4-[*N'*,*N'*-bis(2-chloroethyl)amino]phenyl] acetamide (11) and *N*-[4-[*N'*,*N'*-bis(2-chloroethyl)amino]phenyl]trifluoroacetamide (12) as prodrugs for ADEPT procedures, the chemical stabilities of substrates *N*,*N*-bis(2-chloroethyl)aniline (5) and *N*,*N*-bis(2-chloroethyl)-4-aminoaniline (10) as well as (11) and (12) were determined at pH 7.4 and 37°C. These reactions were carried out in 10^{-3}M phosphate buffer containing 3% (v/v) DMSO to solubilise the mustard substrate. The reactions were followed by two procedures, HPLC assay and ion chromatography, and rates of decomposition were determined.

2.3.1 HPLC assay

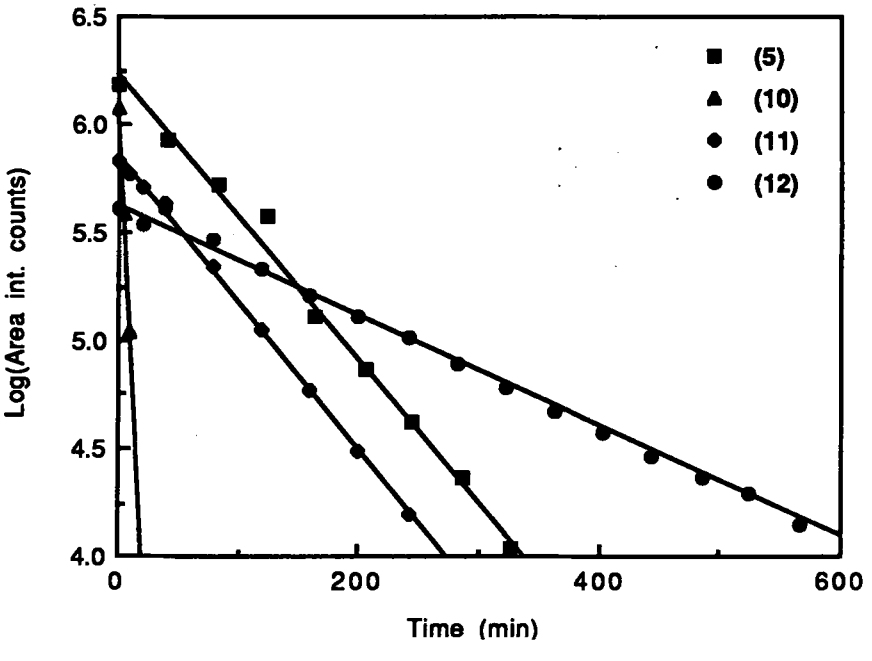
These reactions were carried out in 10^{-3}M phosphate buffer containing 3% (v/v) DMSO at pH 7.4 and 37°C using *ca.* 0.15 mM substrate (Experimental section 5.4.3.3). The reactions were followed by monitoring the decrease in substrate concentration by reverse phase HPLC according to the conditions described in ***Table 5.3*** (Experimental, Section 5.4.3.2). This table also lists the HPLC retention times (Rf) for substrates and hydrolysis products for the assay conditions. In reverse phase column the most polar di-hydroxy compounds elute earlier, followed by the mono-hydroxy compound (half-mustard) and finally the intact mustard. In practice, aliquots (20 μl) of the reaction solution were taken at timed intervals using an autosampler and on elution, the area of the substrate peak was determined by the computing integrator. The integrated peak areas decreased steadily with time to eventually reach a zero value.

The decomposition was assumed to follow *pseudo* first-order kinetics (Equation. 1.1 to 1.5), and k_1 (the constant for the first step) was evaluated from plots of log (peak area) or (peak height) *versus* time. These were reasonably linear, validating equation 1.2. Values of k_1 were accurate to ± 10%.

The variation of both integrated peak areas (or peak heights) and their logarithmic values with time are shown in ***Figure 2.2***. The values of k_1 obtained are summarised in ***Table 2.4***. It is evident that aryl mustard (10) bearing the 4-NH_2 substituent (the potential drug) is *ca.* 15-fold and *ca.* 42-fold more unstable (*ie.* more reactive towards H_2O) than the potential prodrugs (11) and (12), respectively.



a) Disappearance of substrates: **(5)**, Rf=20.47 min; **(10)**, Rf=20.61 min; **(11)**, Rf=24.77 min; **(12)**, Rf=29.80 min



b) Log (disappearance of substrates)

Figure 2.2 Decomposition of aryl mustards (5), (10), (11) and (12) in phosphate buffer at pH 7.4 and 37°C, followed by HPLC: [Buffer]=10^{-3}M in 3% (v/v) aqueous DMSO; [substrate]$_{init.}$ ca. 0.15 mM

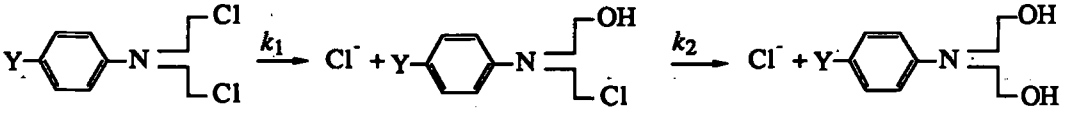
Table 2.4

Values of k_1 for the decomposition of aryl mustards (5), (10), (11) and (12) in phosphate buffer at pH 7.4 and 37°C, followed by HPLC: [Buffer]=10^{-3}M in 3% (v/v) aqueous DMSO; [substrate]$_{init.}$ ca. 0.15 mM

Compound	4-Substituent	$10^4 k_1$ s^{-1}	k_1^X/k_1^H	log (k_1^X/k_1^H)
(5)	H	1.10	1	-
(10)	NH_2	17.35	15.8	1.20
(11)	$NH(CO)CH_3$	1.13	1.03	0.01
(12)	$NH(CO)CF_3$	0.42	0.38	- 0.42

2.3.2 Ion Chromatographic assay

In the ion exchange chromatography method the release of Cl^- ion was measured and plotted as a function of time. Because Cl^- is released in 2 steps (*Scheme 2.8*), its variation with time is complicated and the rate obtained is a complex function of k_1 and k_2 which can be solved by time ratio methods.[99] Since k_1 is known from the HPLC assay (Section 2.3.1), it should be possible to deduce k_2 from the ion chromatography (Cl^-) results as outlined in *Scheme 2.8*.



Scheme 2.8 Two steps involved in the hydrolysis of the aryl mustards

The hydrolysis conditions were identical for both HPLC and ion exchange chromatography assay methods (i.e, phosphate buffer (10^{-3}M), 3% DMSO, pH 7.4 and 37°C) and both were performed using an automatic sample extraction and injection (Experimental Section 5.4.3). For the ion exchange method, however, technical problems prevented stirring of the reaction solution as for the HPLC method. *Figure 2.3* shows the behaviour of these mustards with time.

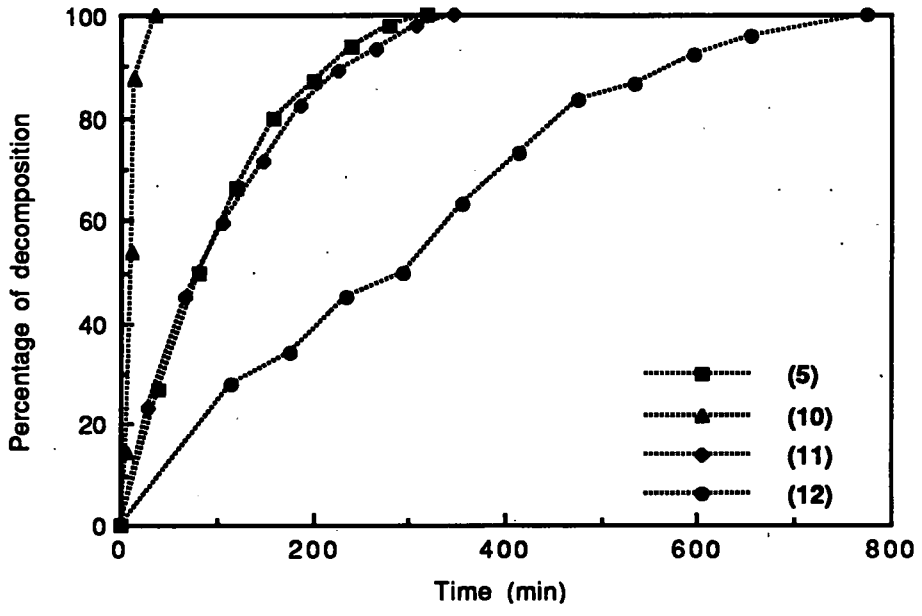


Figure 2.3 Comparison of hydrolysis of mustards (5), (10), (11) and (12) in phosphate buffer at pH 7.4 and 37°C followed by ion exchange chromatograpy: [Buffer] $10^{-3}M$ in aqueous DMSO (3%,v/v); [substrate]$_{init}$ ca. 0.15 mM

Table 2.5 shows the k_1 and k_2 values calculated by the time ratio method (using initial k_1 values obtained in Section 2.3.1).

Table 2.5

Values of k_1 and k_2 for decomposition of aryl mustards (5), (10), (11) and (12) in phosphate buffer at pH 7.4 and 37°C followed by ion exchange chromatography: [Buffer] $10^{-3}M$ in aqueous DMSO (3% v/v); [substrate]$_{init}$ ca. 0.15 mM

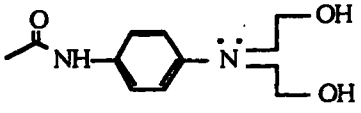
Compound	4-Y Substituent	$10^4 \times k_1$ (s^{-1})	$10^4 \times k_2$ (s^{-1})
(5)	H	2.56	1.79
(10)	NH_2	26.6	16
(11)	$NH(CO)CH_3$	2.9	1.6
(12)	$NH(CO)CF_3$	0.86	0.6

2.4 Isolation and identification of products for the decomposition (hydrolysis) of aryl mustards (11) and (12) in aqueous DMSO at 37°C and different pH

The products were obtained by dissolving the aryl mustard (either 20 mg (11) or 23 mg (12), 0.072 mmol) in aqueous DMSO (7:3, v/v) (25 cm^3) at pH 2, 7 or 12 (pH adjusted with either HCl or NaOH), and heating the solution at 37°C over 24h. The solution was then neutralized with NaOH or HCl and freeze-dried. The residue was extracted with ethyl acetate (20 cm^3) and after vacuum evaporation of the solvent, analysed by MS, GC and GC-MS procedures. When a relatively pure product was obtained, it was also examined by ^{1}H-NMR spectroscopy.

2.4.1 Aryl mustard (11) at pH 2 and 7

These reactions gave a single, major product. The EI mass spectrum showed m/z=238 and the ^{1}H-NMR spectrum in (CD_3OD) showed δ=2.1 (3H, s, $NHCOCH_3$), 3.4 (1H, br s, N*H*), 3.5 and 3.6 (8H,dt, J=5.1 Hz, CH_2), 4.8 (2H, br s, O*H*), 6.6-7.3 ppm (4H, ABqu, J=10.4 Hz, Ar*H*). These data are consistent with structure (59) for this compound.



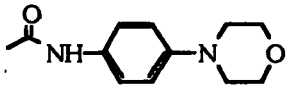
(59)

Chart 5

The GC-MS spectra were inconclusive because (59) is not ameniable to GC assay.

2.4.2 Aryl mustard (11) at pH 12

This reaction gave a mixture of two products. The EI mass spectrum showed parent ions at m/z=238 [M^+ for (59)] and m/z=220 [M^+ for (60)]. The GC-MS assay showed a large peak at Rf=15 min with m/z=220 corresponding to M^+ for (60).



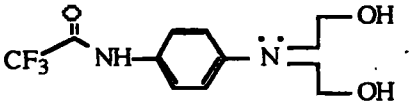
(60)

Chart 6

The above observations were confirmed by GC assays of the reaction mixture against authentic compounds 4-phenylmorpholine (45), 4-morpholinophenylacetamide (60), 4-morpholinoaniline (61), 4-acetamidophenol (62), diethanolamine (63) and 4-aminophenol (64). None of these compounds were evident at pH 2 and 7, but (60) was apparent after 24 min for decomposition at pH 12.

2.4.3 Aryl mustard (12) at pH 2 and 7

These reactions also gave a single, major product. The FAB mass spectrum (positive mode) gave m/z=293 (100%) which corresponds to the MH^+ ion for compound (65).

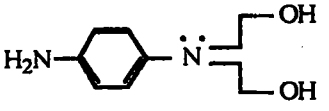


(65)

Chart 7

2.4.4 Aryl mustard (12) at pH 12 , 13 and 14

At pH 12 this reaction gave a mixture of two products. The EI mass spectrum showed two parent ions at m/z=292 [M^+ for (65)] and m/z=196 [M^+ for (66)] at relative intensities of 21% and 12%, respectively.



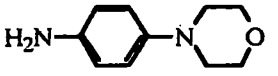
(66)

Chart 8

At pH 13, three products are formed. The EI mass spectrum shows the above two parent ions at m/z=292 [M^+ for (65)] and m/z=196 [M^+ for (66)], plus a third at m/z=178 [M^+ for 4-phenylmorpholine (61)].

At pH 14, there appears to be only one final product. Both ^{1}H-NMR (CD_3OD) examination showing δ=2.8 (4H, t, J=5.5 Hz, CH_2), 3.7,(4H, t, J=5.5 Hz, CH_2), 4.7 (2H, s, NH_2) and 6.6 ppm (4H, J=5.2 Hz, ArH) and the MS(EI) spectrum showing m/z=178 [20%, M^+ for (61)] and 120 (52, $M^+ - C_3H_6O$) are consistent with the structure (61) for this product.

NH_2) and 6.6 ppm (4H, J=5.2 Hz, ArH) and the MS(EI) spectrum showing m/z=178 [20%, M^+ for **(61)**] and 120 (52, M^+ - C_3H_6O) are consistent with the structure **(61)** for this product.



(61)

Chart 9

2.5 Decomposition (hydrolysis) of aryl mustards (10), (11) and (12) in aqueous DMSO at 37°C and different pH followed by HPLC

In a standard procedure, the substrate mustard in neat DMSO (50 μl, $9x10^{-2}$M) was injected into a thermostatted reaction vessel (37°C) containing aqueous DMSO (30 cm^3, 7:3, v/v) adjusted to the required pH by the addition of either NaOH (1M) or HCl (1M). The concentration of substrate in the reaction solution was *ca.* $1.5x10^{-4}$M. The reaction mixture was stirred and at timed intervals, an aliquot (20μl) was taken by an auto sampler and injected into the HPLC analyser. Details of the HPLC assay including retention times (Rf) for substrates and products are given in ***Table 5.3*** (Experimental Section 5.4.3.2 and 5.4.3.4). Aliquots were collected at appropriate times, according to the kinetics of each substrate. Each aliquot was eluted to display all of the major products as well as the substrate. The area of each peak was recorded by a computing integrator and subsequently, either the peak heights or the integrated peak areas for the substrate and each product were plotted with respect to time. From these data for the substrate, the *pseudo* first-order rate coefficient k_1 (equation 1.2) for the first hydrolysis step, was determined from plots of log peak area or log peak height *versus* time. These plots were invariably linear.

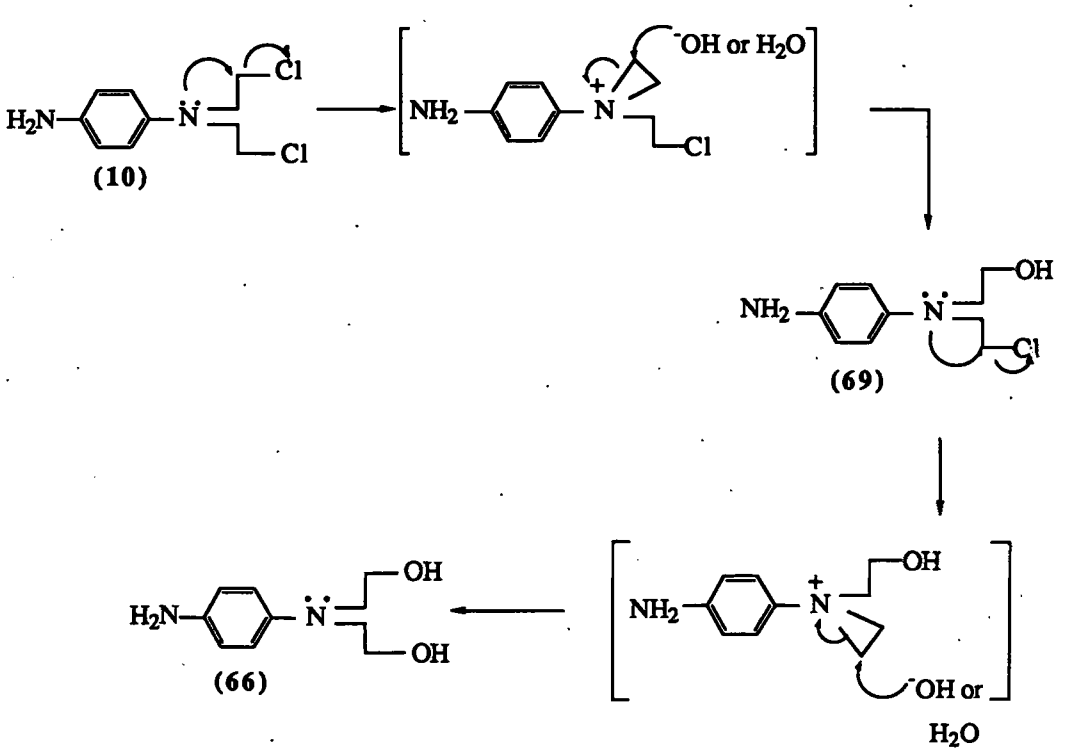
For hydrolysis of compounds **(11)** and **(12)** at pH 2, values of both k_1 and k_2 were calculated by substitution of concentrations of the mustard (A), the half-hydrolysed mustard (B) and the final diol product (C) (***Scheme 1.12***) into the appropriate first-order equation reaction (1.2 to 1.5) and optimized using a mathematical method to determine the best fit (Enzeffiter method).[159] Formation of several final products in basic conditions prevent the determination of values of k_1 and k_2, simultaneously.

2.5.1 Aryl mustard (10)

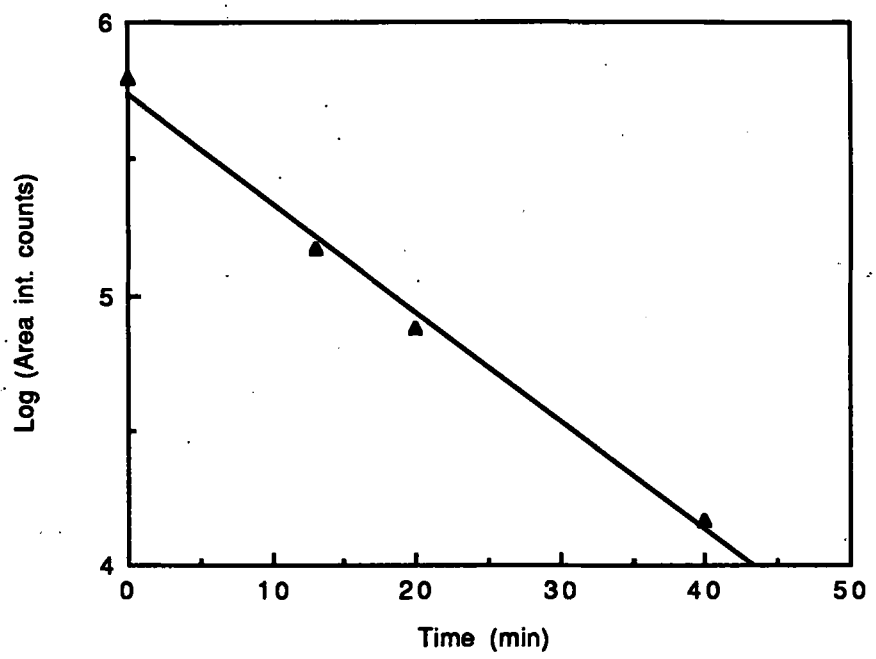
Because of a lack of time and purification difficulties, *N,N*-bis(2-chloroethyl)-4-amino-aniline **(10)** was examined at pH 12 only.

The HPLC data summarised in ***Figure 2.4*** show that **(10)** (Rf=20.61 min) disappears rapidly in *ca.* 20 min. The HPLC trace after 40 min showed no evidence of **(10)**. The reaction mixture rapidly darkened and the major product (Rf=4.20 min) was identified by HPLC comparison against authentic material, and by MS assay of the isolated reaction product, as *N,N*-bis(2-hydroxyethyl)-4-amino-aniline **(66)**. Compound **(66)** was unstable, reaching a maximum concentration at *ca.* 40 min, then decomposed steadily. It was also possible to detect at Rf=14.64 min, the half-hydrolysed mustard **(69)** in the conversion of **(10)** to **(66)**. Compound **(69)** showed a high turnover and was observable only in aliquots at 13 min and 20 min reaction, which were frozen immediately on collection and subsequently analysed.

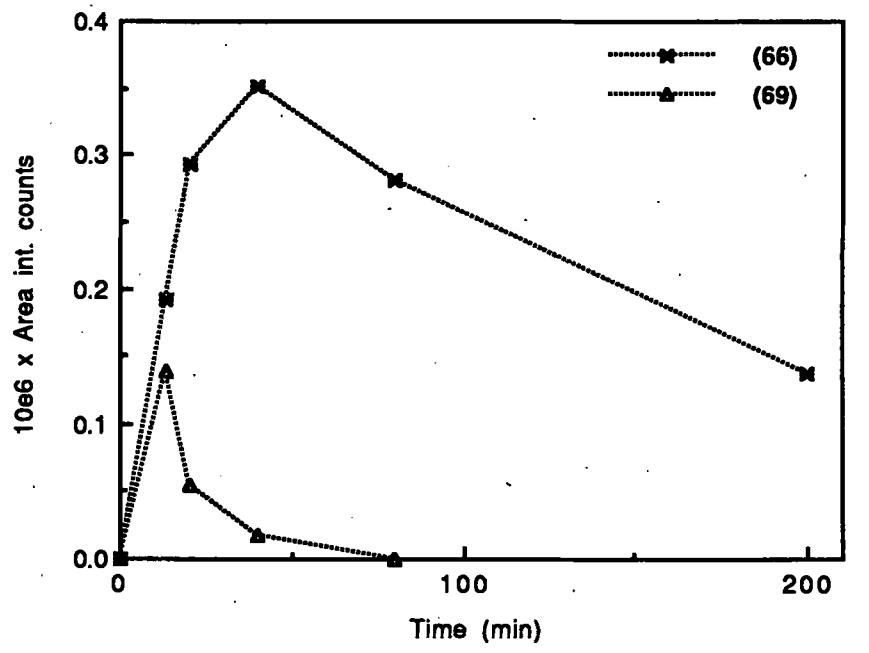
The plot of log (peak area) for **(10)** *versus* time gave an approximate value of $k_1 = 4000 \times 10^{-5}\ s^{-1}$ *ie ca.* 120-fold faster than the decomposition of **(12)** (Section 2.5.3.2) and 880-fold faster then the decomposition of **(11)** (Section 2.5.2.2), under similar conditions. The decomposition pathway for **(10)** at pH 12 is outlined in ***Scheme 2.9***.



Scheme 2.9 Decomposition pathway for N,N-bis(2-chloroethyl)-4-amino-aniline (10) in aqueous DMSO (7:3, v/v) at pH 12 and 37°C



a) Decomposition of starting material (10), Rf=20.61 min



b) Formation of decomposition products: (66), Rf=4.2 min; (69), Rf=14.64 min

Figure 2.4 HPLC assays of N,N-bis(2-chloroethyl)-4-amino-aniline (10) decomposition in aqueous DMSO (7:3, v/v) at pH 12 and 37 °C: $[10]_{init}$ ca. 0.15 mM

Hydrolysis is envisaged to proceed via two sequential S_ni reactions producing aziridinium ion intermediates which react with water or HO^-, as for aryl mustards **(11)** (Section 2.5.2.3) and **(12)** (Section 2.5.3.4). Interestingly, concurrent formation of 4-morpholinoaniline appears not to proceed with **(10)** at pH 12. It is possible that activation by 4-amino substituent promotes aziridinium ion formation by the intermediate **(69)** rather than reaction of the hydroxyl moiety to give 4-morpholinoaniline.

2.5.2 Aryl mustard (11)

2.5.2.1 pH 2

The HPLC data summarised in ***Figure 2.5*** show that: 1), the peak height of the substrate *N*-[4-[*N'*,*N'*-bis(2-chloroethyl)amino]phenyl]acetamide **(11)** at Rf=24.77 min, decreases to zero over 1000 min; 2), the peak area at Rf=17.87 min, corresponding to the half-hydrolysed mustard **(67)**, increases to a maximum at *ca.* 300 min, then decreases to a small value at 1500 min; 3), a major product corresponding to 75% reaction, at Rf=8.63 min, increases over 1000 min after a clear induction period. This major product was identified as *N*-[4-[*N'*,*N'*-bis(2-hydroxyethyl)amino]phenyl]acetamide **(59)**, by HPLC coelution with authentic material. The identity of this product after neutralization of the reaction mixture, freeze-drying of the solution and extraction of the residue with ethyl acetate, confirmed the product identity (see above).

The variation of log (peak height) for **(11)** with time is linear (see ***Figure 2.5 a***) and gives $k_1 = 2.65 \times 10^{-5}\ s^{-1}$.

2.5.2.2 pH 12 and pH 13

The HPLC data in ***Figure 2.6*** for pH 12 show similar time-dependent behaviour for the peak height of the substrate **(11)** and the peak area of the half-hydrolysed mustard **(67)**. The chromatograms also showed the formation of two main products. The major one, at Rf=8.63 min (corresponding to 74% reaction) is *N*-[4-[*N'*,*N'*-bis(2-hydroxyethyl)amino] phenyl] acetamide **(59)**, as found at pH 2. The second product at Rf=15.16 min (corresponding to 20% reaction) was identified as 4-morpholinophenylacetamide **(60)**, by HPLC coelution with authentic material. The identity of this product was also confirmed by GC-MS of isolated material (see above).

As for pH 2, the variation of log (peak height) for **(11)** with time, is linear (see ***Figure 2.6 a***) and gives $k_1 = 4.53 \times 10^{-5}\ s^{-1}$.

The reaction at pH 13 was very similar to pH 12 except for a different ratio of the two hydrolysis products, and a different rate. Thus, *N*-[4-[*N'*,*N'*-bis(2-hydroxyethyl)amino] phenyl]acetamide **(59)** was the minor product in 20% yield, whereas 4-morpholino-phenylacetamide **(60)** was obtained in 75% yield. The value of k_1 is 8.0×10^{-5} s^{-1}.

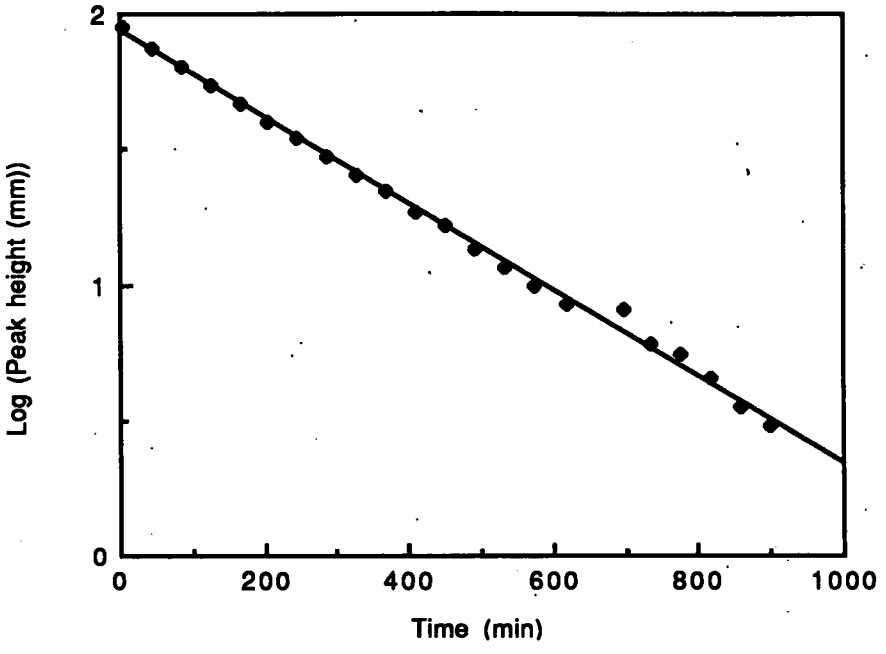
2.5.2.3 Summary

The effect of pH on reaction rates (rate for the first step of hydrolysis) and products is summarised in ***Table 2.6.*** The products are consistent with the pathways outlined in ***Scheme 2.10.*** At pH 2, hydrolysis seems to proceed via two sequential S_Ni reactions producing aziridinium ion intermediates which react with water or HO^-. At pH 12 and 13, the reaction is complicated by ionization of the half-hydrolysed mustard, which promotes an alternative S_Ni reaction leading to the formation of N-[4-(4-morpholinyl)phenylacetamide **(60)**. The alternative reaction is pH dependent and the yield of **(60)** increases from 20% to 75% with increase of pH from 12 to 13. The rate of decomposition of **(11)** is virtually independent of pH. This supports an S_Ni reaction for the first step of ***Scheme 2.10*** rather than direct attack by H_2O or HO^-.

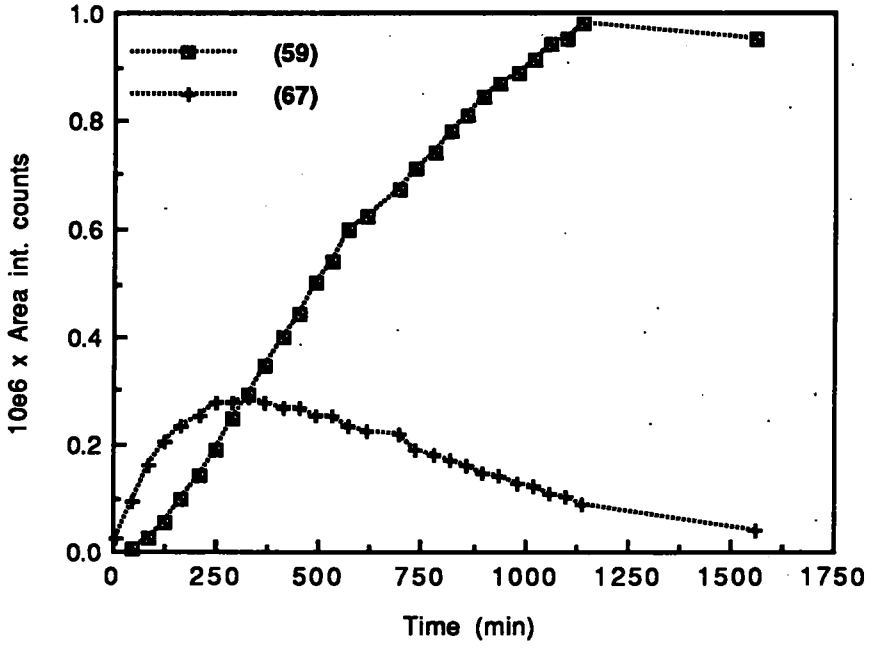
Table 2.6

Dependence of k_1 and products on pH for N-[4-[N',N'-bis(2-chloroethyl)amino phenyl]acetamide (11) decomposition in aqueous DMSO (7:3,v/v) at 37°C

pH	Product / Yield (%)	$10^5 \times k_1$ (s^{-1})
2	**(59)** / 75	2.65
12	**(59)** / 74 **(60)** / 20	4.53
13	**(59)** / 20 **(60)** / 75	8.0

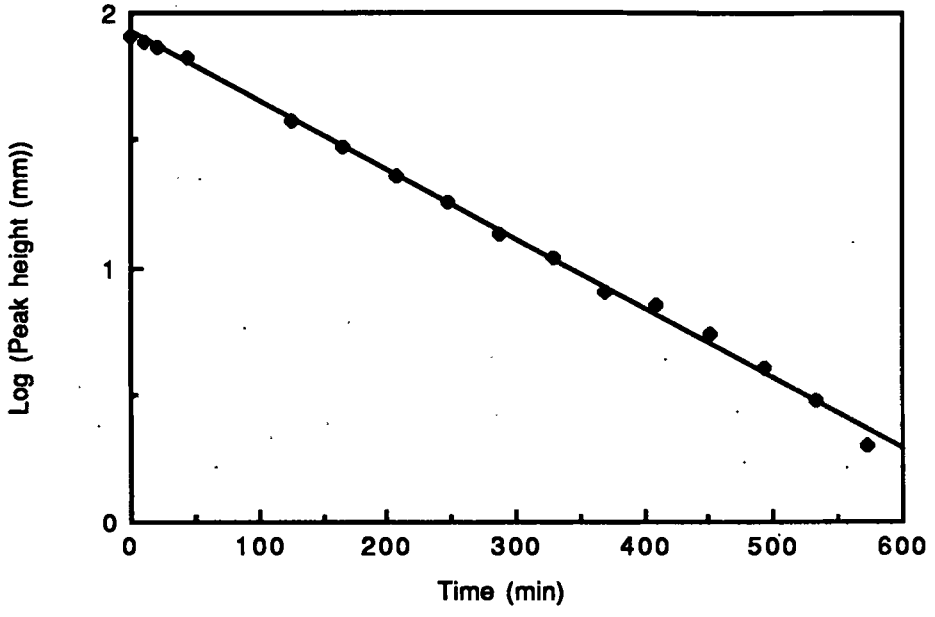


a) Disappearance of starting material (11), Rf=24.77 min

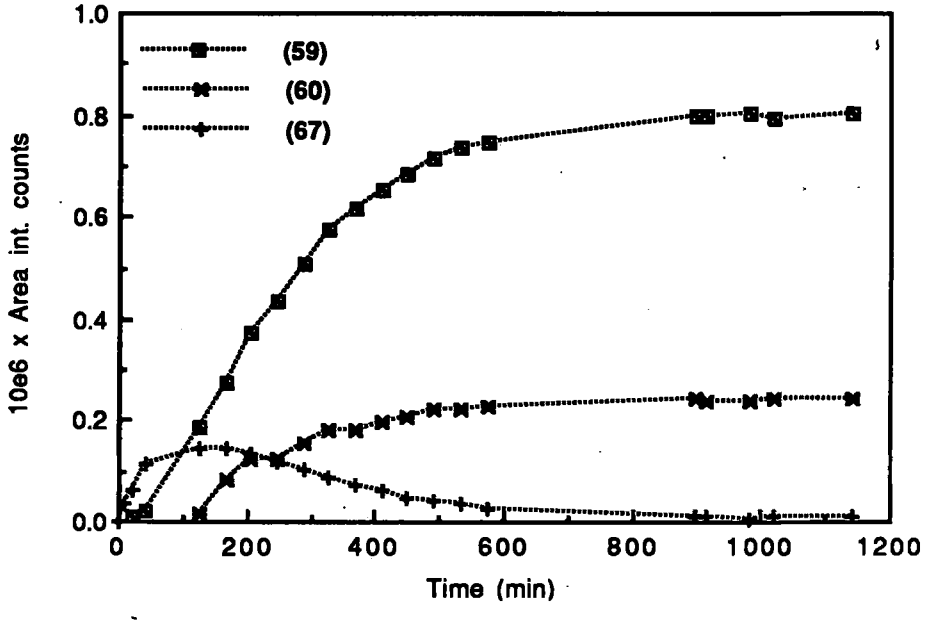


b) Formation of products: (59), Rf=8.63 min and (67), Rf=17.87 min

Figure 2.5 *HPLC assays of N-[4-[N',N'-bis(2-chloroethyl)amino]phenyl]acetamide (11) decomposition in aqueous DMSO (7:3, v/v) at pH 2 and 37°C: $[11]_{init}$ ca. 0.15 mM*

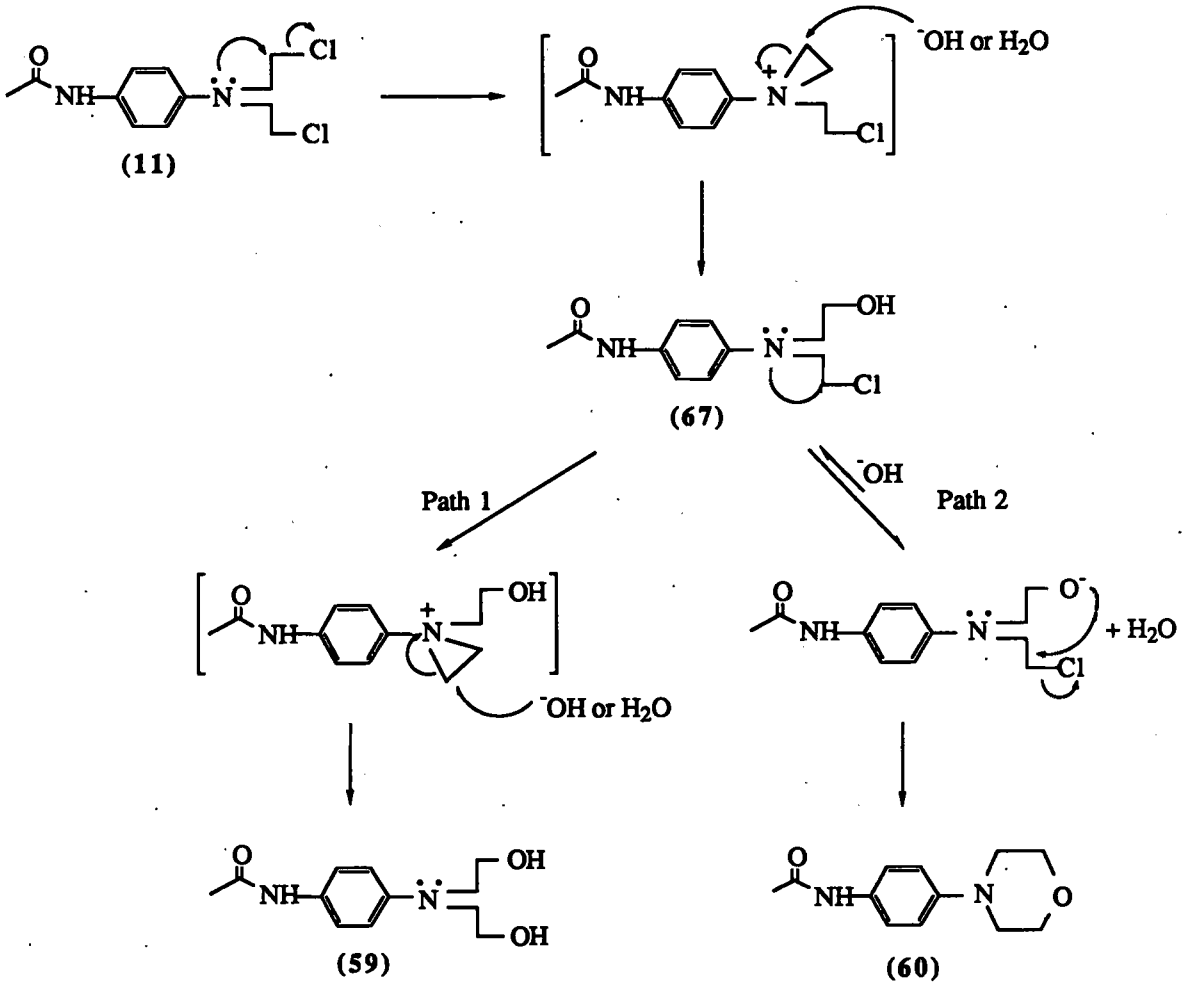


a) Disappearance of starting material (11), Rf=24.77 min



b) Formation of products: (59), Rf=8.63 min; (60), Rf=15.16 min; (67), Rf=17.87 min

Figure 2.6 HPLC assays of N-[4-[N',N'-bis(2-chloroethyl)amino]phenyl]acetamide (11) decomposition in aqueous DMSO (7:3,v/v) at pH 12 and 37°C: [11] $_{init}$ ca. 0.15 mM



Scheme 2.10 Decomposition pathways for N-[4-[N',N'-bis(2-chloroethyl)amino]phenyl] acetamide (11) in aqueous DMSO (7:3,v/v) at 37°C and different pH

2.5.3 Aryl mustard (12)

2.5.3.1 pH 2

The HPLC data summarised by ***Figure 2.7*** is very similar to that obtained for **(11)**. Thus, the peak area of the substrate [*N*-[4-[*N'*,*N'*-bis(2-chloroethyl)amino]phenyl]trifluoroacetamide **(12)** at Rf=29.8 min decreases to zero over 3000 min, while the peak area at Rf=23.61 min corresponding to the half-hydrolysed mustard **(68)**, increases to a maximun value at *ca.* 500 min, and then decreases to virtually zero at 3000 min. Also a single major product corresponding to 90% reaction with Rf=17.75 min increases steadily after an induction period. This product was identified as *N*-[4-[*N'*,*N'*-bis(2-hydroxyethyl)amino]phenyl]trifluoroacetamide **(65)**, by HPLC coelution with authentic material. The identity of this product,

after neutralization of the reaction mixture, freeze-drying of the solution and extraction of the residue with ethyl acetate, confirmed the product identity (see above).

The variation of log (peak area) for the disappearance of **(12)** with time is reasonably linear (see ***Figure*** *2.7 a*) and gives $k_1 = 9.8 \times 10^{-6}\ s^{-1}$.

2.5.3.2 pH 12

The HPLC data in ***Figure*** *2.8* for pH 12 shows similar behaviour for the peak areas of substrate **(12)**, although its decomposition is very much faster and virtually complete in 100 min. Also, the half-hydrolysed substrate **(68)** is observable, but its peak area reaches a maximum at *ca.* 50 min and has disappeared at *ca.* 110 min. As for substrate **(11)**, the chromatograms show formation of two main products at pH 12. One product at Rf=17.75 min, that forms faster and decomposes further, is identical to the N-[4-[bis(2-hydroxyethyl) amino]phenyl]trifluoroacetamide **(65)** formed at pH 2. The second product, at Rf=4.20 min corresponding to 69% reaction at *ca.* 110 min, was identified as *N,N*-bis(2-hydroxyethyl)-4-aminoaniline **(66)** by comparison of HPLC retention times against authentic material and by MS examination of isolated product on completion of the reaction (see above). The decrease of **(66)** with time probably relates to its oxidation to 4-benzoquinone di-imine and subsequent hydrolysis.[158]

The variation of log (peak height) for **(12)** with time (see ***Figure*** *2.8 a*) gave k_1 ca. $3.25 \times 10^{-4}\ s^{-1}$.

2.5.3.3 pH 13

The HPLC data for decomposition under more alkaline conditions (***Figure*** *2.9*) shows faster reaction and an increased number of products. The substrate **(12)** peak virtually disappears after 36 min and is replaced by a series of peaks for products that form and decompose in the early stages of the reaction. A peak at Rf=23.61 min corresponding to the half-hydrolysed mustard **(68)** for the conversion of **(12)** into **(65)** is just detectable (< 3% peak area relative to initial peak area of substrate **(12)**) in one aliquot at 12 min, and a second peak at Rf=14.70 min probably due to the half-hydrolysed mustard **(69)** for the conversion of **(10)** into **(66)**, is only seen over the initial 36 min. Two other peaks at Rf=17.75 min (identified as *N*-[4-[*N'*,*N'*-bis(2-hydroxyethyl) amino]phenyl]trifluoroacetamide **(65)**) and Rf=20.61 min (identified as *N,N*-bis(2-chloroethyl)-4-amino-aniline **(10)**) are also evident in the HPLC chromatograms in small amounts (< 3%, relative to initial peak area of substrate **(12)**) for the early stages of the reaction only (< 200 min). Two peaks at Rf=4.20 and 12.18 min, corresponding to more stable products which increase at longer reaction times were identified as *N,N*-bis-

(2-hydroxyethyl)-4-amino-aniline (66) and 4-morpholinoaniline (61), respectively. The identification of all these products apart from the half-hydrolysed mustards (68) and (69) was made by HPLC retention times against authentic compounds.

The variation of log (peak height) for (12) with time (see *Figure 2.9 a*) gave k_1 ca. 505 s^{-1}.

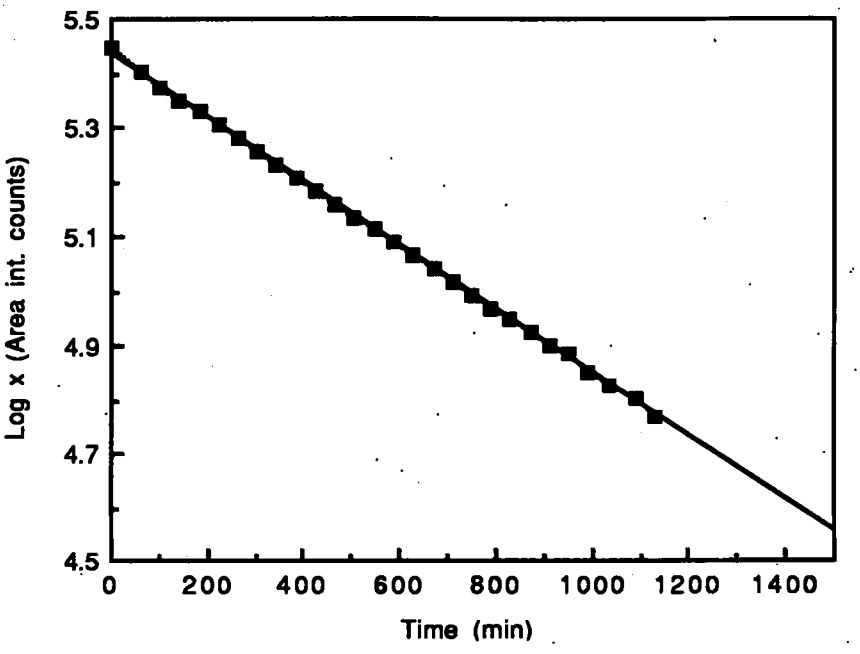
2.5.3.4 Summary

The effect of pH on reaction rates and products for the decomposition of [*N*-[4-[*N'*,*N'*-bis-(2-chloroethyl)amino]phenyl]trifluoroacetamide (12) in aqueous DMSO (7:3,v/v) at 37°C is summarised in *Table* 2.7. For both compounds (11) and (12), decomposition is slower at pH 2 than at pH 12 and 13. While at pH 2 the decomposition is ca. 3-fold faster for (11) than for (12), at pH 12 and 13 an inverse situation occurs: (12) hydrolyses ca. 7-fold faster then (11) and, in contrast to (11), cleavage of the N-trifluoroacetyl moiety of (12) is apparent at pH 12 and 13. These findings suggest that different decomposition pathways operate in acidic and basic conditions. These pathways, outlined in *Scheme 2.11*, have the following characteristics:

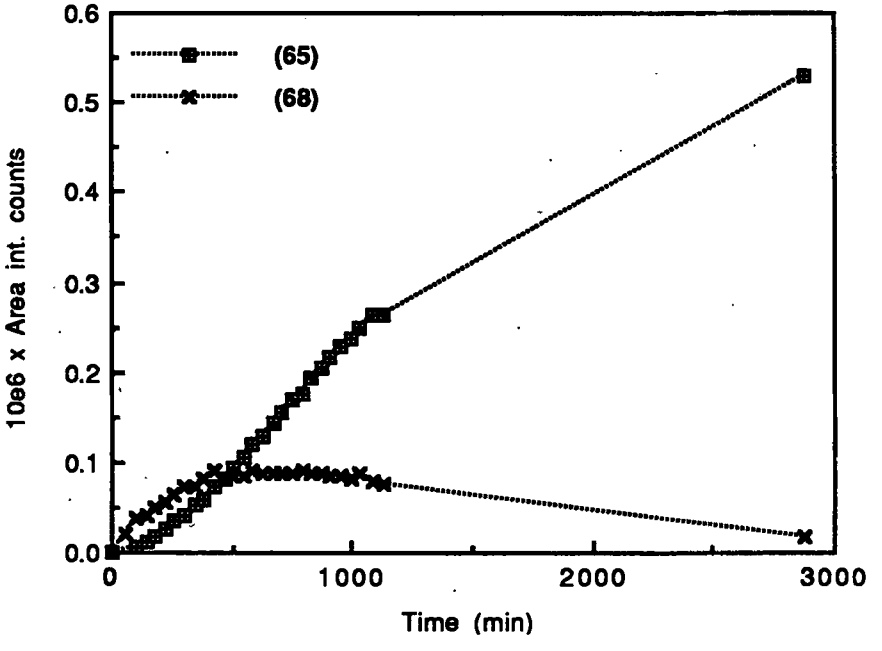
1) At pH 2, the formation of *N*-[4-[*N'*,*N'*-bis(2-hydroxyethyl)amino]phenyl] trifluoroacetamide (65) from (12) seems to involve similar, sequential S_ni reactions producing aziridinium ion intermediates as for (11) (*Scheme 2.10*). The rate of this reaction is slightly slower, presumably because of increased electron-withdrawal by the *N*-trifluoro acetyl substituent (in (12)) compared with N-acetyl (in (11)). This reaction is described by *Path 1* of *Scheme 2.11.*

2) At pH 12, formation of *N*-[4-[bis(2-hydroxyethyl)amino]phenyl]trifluoro-acetamide (65) proceeds via *Path 1*, identical to that for the decomposition of (11). Formation of *N,N*-bis(2-hydroxyethyl)-4-amino-aniline (66), however, must form by an alternative *Path 2* involving initial cleavage of the *N*-trifluoroacetyl moiety to give (10), which rapidly decomposes to (66) via similar S_ni reactions to those for (11) and (12). Because the overall decomposition of (12) at pH 12 is *ca.* 7-fold faster than (11), formation of (66) via *Path 2* must predominate.

3) At pH 13, decomposition via *Path 2* is complicated by ionization of the half-hydrolysed mustard (69) which promotes an alternative S_ni reaction leading to the formation of 4-morpholinoaniline (61), in addition to (66). Here too, the *ca.* 7-fold faster decomposition of (12) relative to (11) implies (66) and (61) form predominantly by *Path 2.*

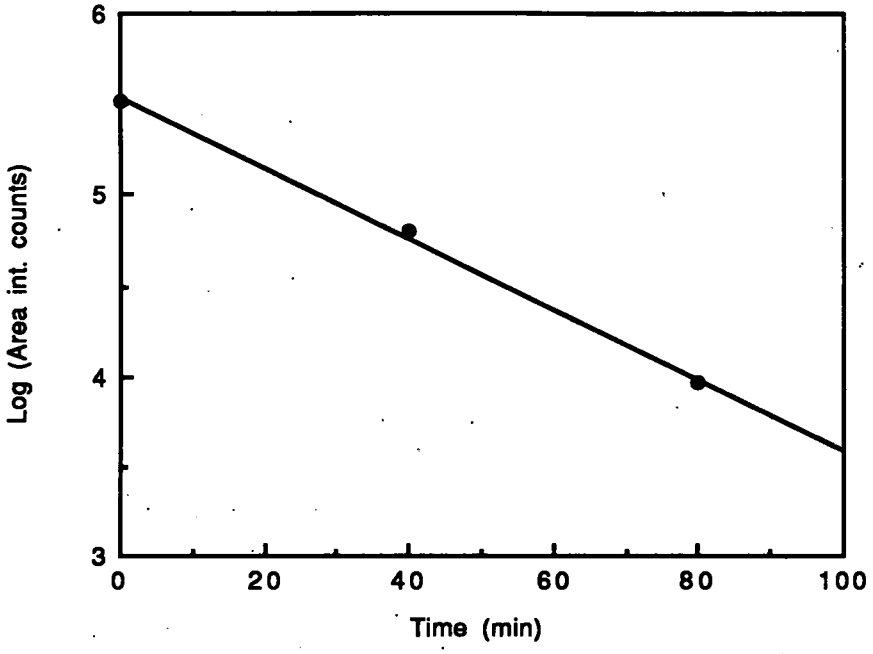


a) Disappearance of starting material (12), Rf=29.80 min

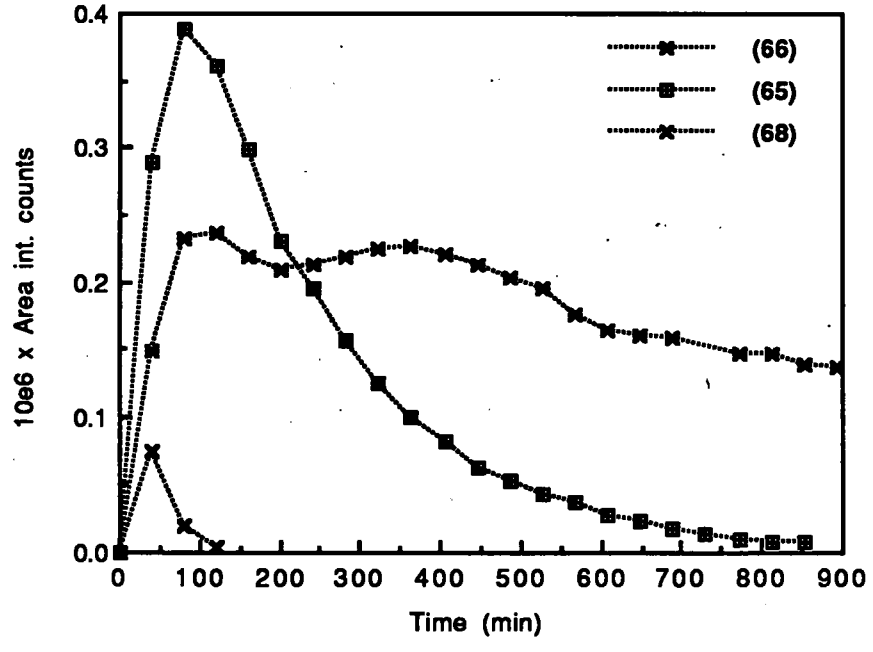


b) Formation of decomposition products: (65), Rf=17.75 min and (68), Rf=23.61 min

Figure 2.7 HPLC assays of [N-[4-[N',N'-bis(2-chloroethyl)amino]phenyl]trifluoroacetamide (12) decomposition in aqueous DMSO (7:3,v/v) at pH 2 and 37°C: $[12]_{init.}$ ca. 0.15 mM

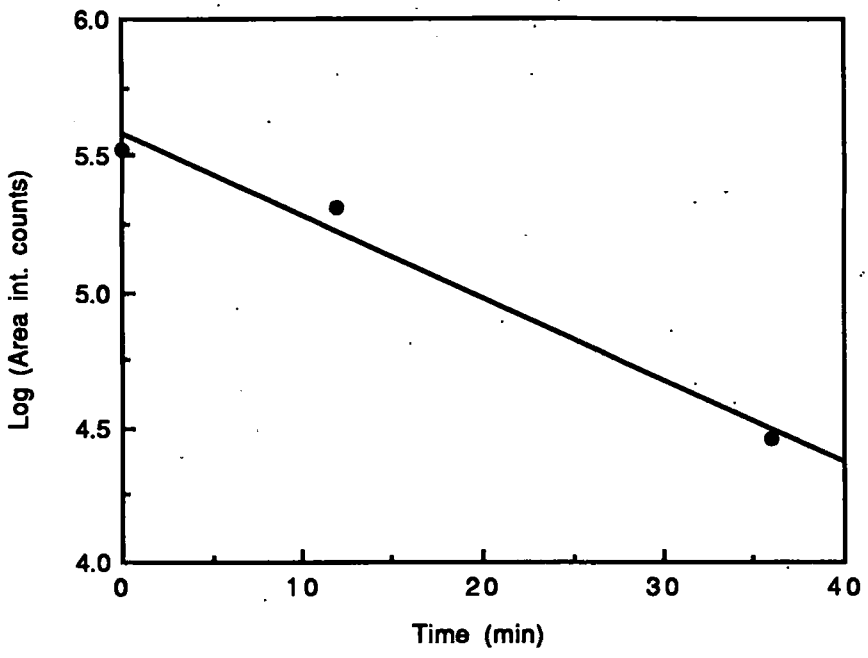


a) Disappearance of substrate (12), Rf=29.80 min

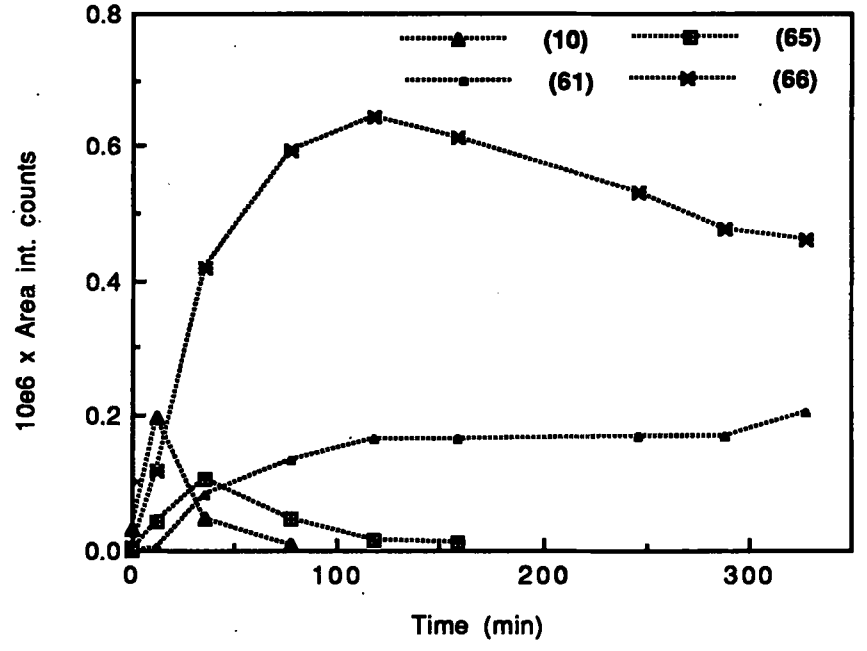


b) Formation of decomposition products: (65), Rf=17.75 min; (66), Rf=4.20 min; (68), Rf=23.61 min

Figure 2.8 HPLC assays of [N-[4-[N',N'-bis(2-chloroethyl)amino]phenyl]trifluoroacetamide (12) decomposition in aqueous DMSO (7:3,v/v) at pH 12 and 37°C: $[12]_{init.}$ ca. 0.15 mM

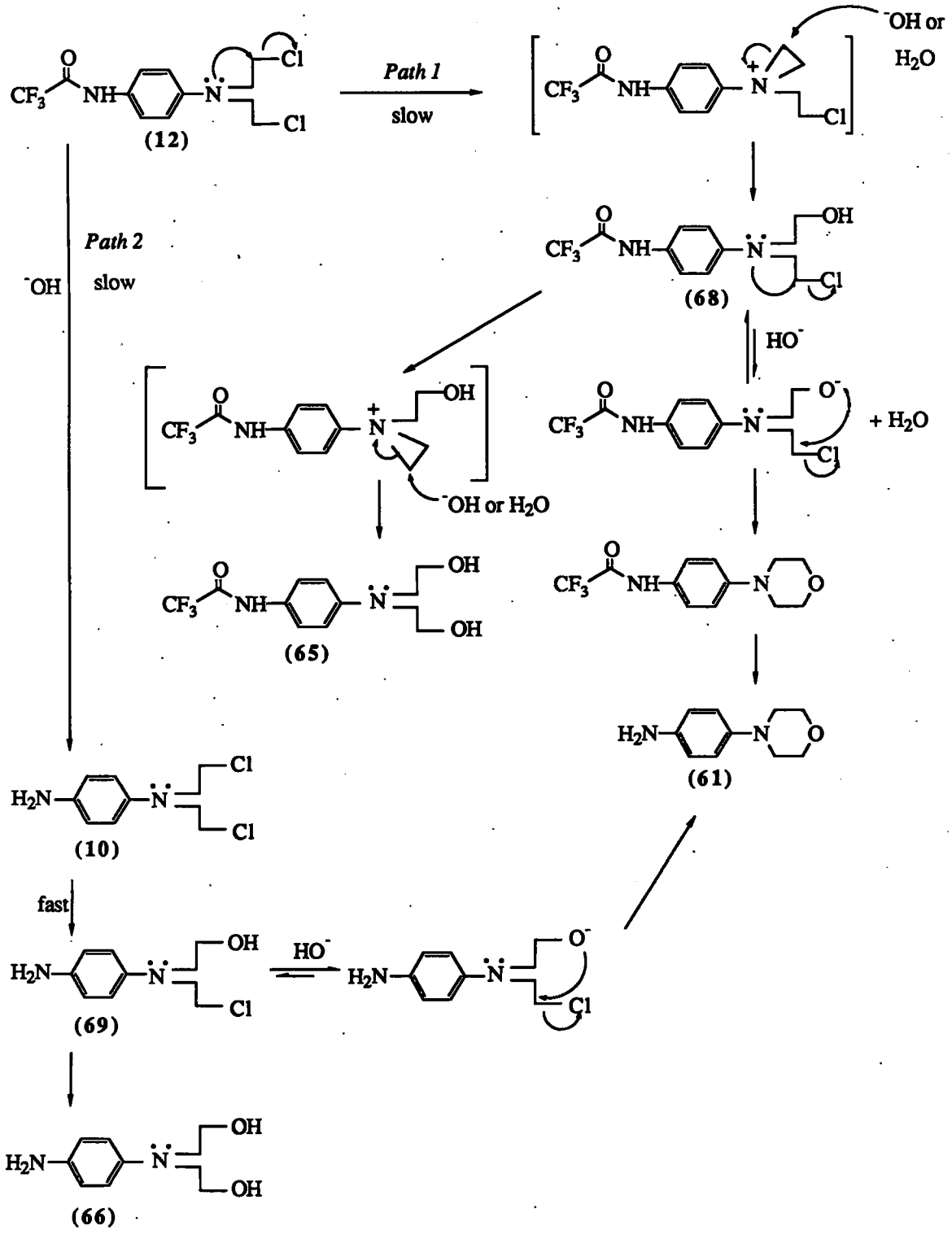


a) Disappearance of starting material (12), Rf=29.80 min



b) Formation of decomposition products: (10), Rf=20.61 min; (61), Rf=12.18 min; (65), Rf=17.75 min; and (66), Rf=4.20 min

Figure 2.9 HPLC assays of [N-[4-[N',N'-bis(2-chloroethyl)amino]phenyl]trifluoroacetamide (12) decomposition in aqueous DMSO (7:3,v/v) at pH 13 and 37°C: $[12]_{init.}$ ca. 0.15 mM



Scheme 2.11 *Decomposition pathways for [N-[4-[N',N'-bis(2-chloroethyl)amino]phenyl] trifluoroacetamide (12) in aqueous DMSO (7:3,v/v) at 37°C and different pH*

Table 2.7

Dependence of k_1 and products on pH for [N-[4-[N',N'-bis(2-chloroethyl)amino]phenyl] trifluoroacetamide (12) decomposition in aqueous DMSO (7:3,v/v) at 37°C

pH	Product / Yield (%)	$10^5 k_1$ (s^{-1})
2	**(65)** / 90	0.98
12	**(65)** / 10	32.5
	(66) / 69	
13	**(66)** / 64	50.5
	(61) / 31	

2.6 Hydrolysis of aryl mustards (10), (11) and (12) in aqueous DMSO at 37°C and different pH followed by UV-visible spectrophotometry

The hydrolysis of *N,N*-bis(2-chloroethyl)-4-aminoaniline **(10)**, *N*-[4-[*N',N'*-bis(2-chloroethyl)amino]phenyl]acetamide **(11)** and *N*-[4-[*N',N'*-bis(2-chloroethyl)amino]phenyl] trifluoroacetamide **(12)** in aqueous DMSO (99:1, v/v, pH 12 and 2) and in phosphate buffer/DMSO (99:1,v/v, pH 7.4) at 37°C was also followed by UV-visible spectrophotometry, recording the spectra between 200 and 400 nm at timed intervals.

In general, the mustard in DMSO (30µl, 1.2×10^{-2}M) was injected into an aqueous solution (3 cm^3) at pH 12 or 2 (adjusted with 1M NaOH or 1M HCl) or 0.05M aqueous phosphate buffer at pH 7.4, contained in a cuvette thermostatted at 37°C. This gave a substrate concentration of *ca.* 1×10^{-4}M. The first spectrum was recorded immediately after addition of the substrate and then at timed intervals.

Some of the UV-Visible spectra were inconclusive; it was not possible to observe appreciable changes between the spectrum of the substrate and the spectrum of the final reaction mixture. The main reason is due to similar λ_{max} and extinction coefficients of the starting material, the intermediate half-hydrolysed mustards and the final products, as summarized in ***Table 5.1*** (Experimental). Further, protonation of some compounds (NH_2 groups or mustard amine groups (pka *ca.* 5.8) complicated the interpretation of spectral changes.

Where substantial spectral changes were observed, an approximate value of the decomposition rate {rate = k_o [substrate]} was calculated from reaction half-lives ($t_{1/2}$)

where $t_{1/2}$ = time for the absorbance of the reaction mixture to be reduced to half of its value ($t_{1/2} = 0.693\ k_o$). Attempts to calculate k_o from plots of log (A_∞ - A_t) versus time, where A_∞ and A_t = absorbances at time t and ∞, respectively, at a wavelength characteristic of the substrate, were unsatisfactory mainly because infinity values were difficult to evaluate.

2.6.1 Aryl mustard (10)

At pH 7, there is no observable change in the spectra over 10h, with a maximum at 260 mn of constant intensity.

At both pH 12 (***Figure 2.10***) and 2 (***Figure 2.11***), there is a decreased intensity of λ_{max}=255 and 260 nm, respectively **(10)** with time, faster at pH 12 (k_o=2.3x10^{-4} s^{-1}) than pH 2 (k_o=2.6x10^{-5} s^{-1}). The final products absorb at *ca.* 230 nm.

2.6.2 Aryl mustard (11)

At pH 7, there was no appreciable change in the UV-Visible spectra over 24h. The λ_{max}= 270 nm of substrate **(11)** and λ_{max}=271 nm of the major final product *N*-[4-[*N'*,*N'*-bis-(2-hydroxy)ethyl]amino]phenyl]acetamide **(59)**, are virtually identical at this pH.

At pH 12, a similar situation applies, although the spectra show a very small decrease of intensity of λ_{max}=270 nm and small increase at λ_{max}=250 nm, with an isosbestic point at 260 nm. These changes probably relate to the formation of the second product, 4-morpholinophenylacetamide **(60)**, with λ_{max}=258 nm. This product is formed only in 20% yield, compared to 77% for **(59)** (with λ_{max}= 271 nm), so the spectral changes are attenuated.

At pH 2, it was possible to observe the decomposition of **(11)** (***Figure 2.12***) and the hydrolysis rate measured from these spectra give k_o=9.6x10^{-5} s^{-1}, about the same as the rate obtained by HPLC analysis (k_o=2.7x10^{-5} s^{-1}). The difference may relate to the higher concentration of DMSO (30%) used for the HPLC analysis. ***Figure 2.12*** shows the decrease in **(11)** (λ_{max}=265 nm) and the increase of **(59)** (λ_{max}=250 nm) with time, and a well-defined isosbestic point at *ca.* 260 nm.

2.6.3 Aryl mustard (12)

At pH 7, only a small variation of λ_{max}=286 nm for **(12)** was observed over 24h. This reflects the similarity of λ_{max} for the substrate and products ((**59**), λ_{max}=271 nm.

At both pH 2 and 12, however, it was possible to observe spectral changes that reflect the decomposition of **(12)** and to relate them to the HPLC results.

Figure 2.13 refers to the decomposition of **(12)** at pH 12. It was a fast reaction with k_o=5.8x10^{-4} s^{-1} compared with k_o=3.25x10^{-4} s^{-1}, for HPLC. There was not a well-defined isosbestic point due to the formation of several products. It was possible however, to observe the rapid decrease of **(12)** (λ_{max}=279 nm) and the rapid formation of **(10)** (λ_{max}=254 nm). Product **(10)** decomposes rapidly and the absorbance above 240 nm virtually disappears. The final products absorb at lower wavelengths [**(66)**, λ_{max}=253 nm; **(61)**, λ_{max}=220 nm].

At pH 2 (*Figure 2.14*) there is a well-defined isosbestic point at *ca.* 260 nm reflecting the conversion of **(12)** (λ_{max}=285 nm) into N-[4-[N',N'-bis(2-hydroxyethyl)amino]phenyl] trifluoroacetamide **(65)** (λ_{max}=248 and 290 nm). A value of k_o=4.05 x 10^{-5} s^{-1} is obtained from these data compared with k_o=0.98 x 10^{-5} s^{-1} by HPLC.

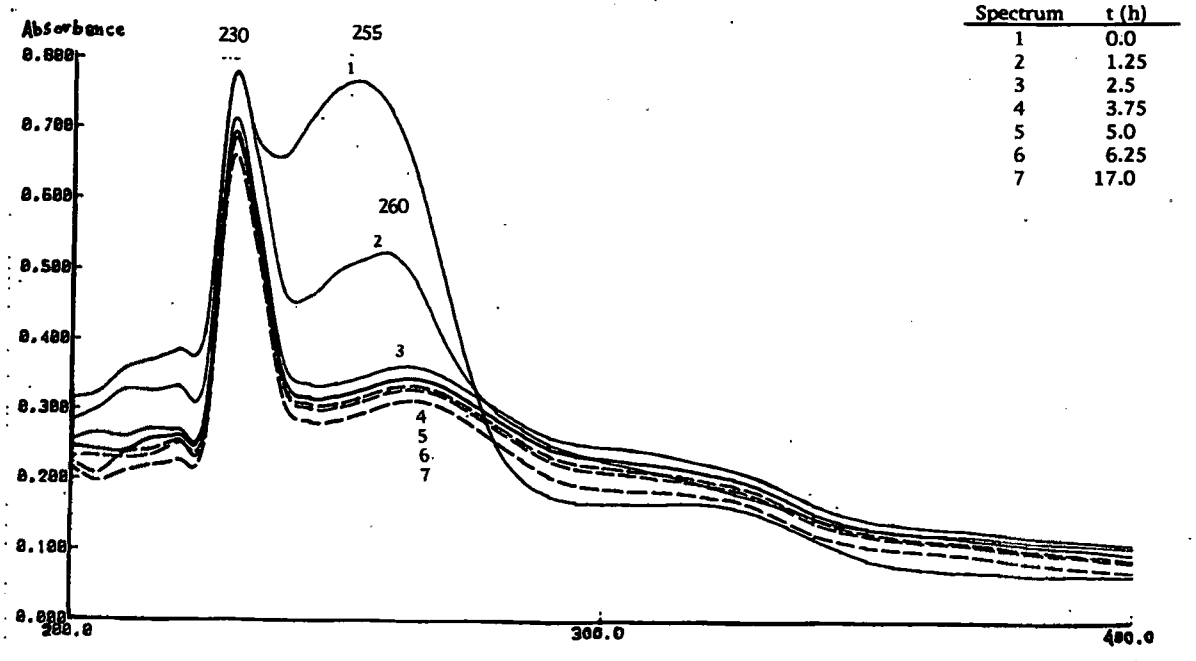


Figure 2.10 *UV/Vis spectra of hydrolysis of N,N-bis(2-chloroethyl)-4-amino-aniline* **(10)** *in aqueous DMSO (9:1,v/v) at 37°C and pH 12 :* **[10]** $_{init.}$ *ca. 1x10^{-4}M*

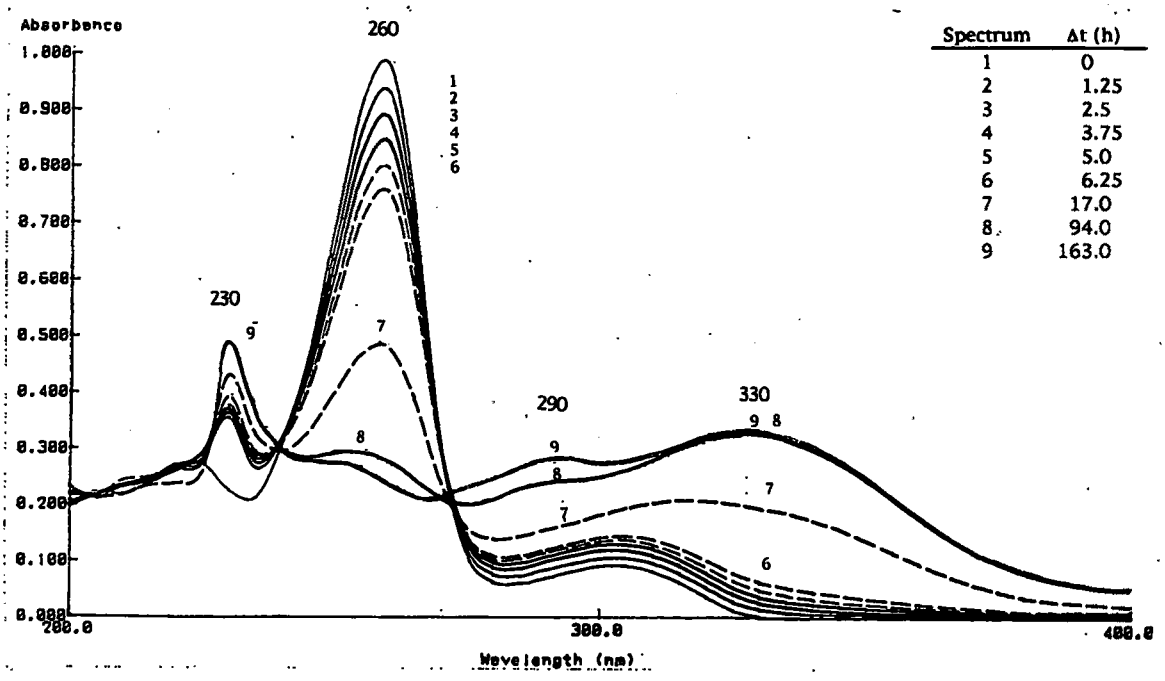


Figure 2.11 *UV/Vis spectra of hydrolysis of N,N-bis(2-chloroethyl)-4-amino-aniline* ***(10)*** *in aqueous DMSO (9:1,v/v) at 37°C and pH 2:* $[10]_{init.}$ *ca.* $1x10^{-4}M$

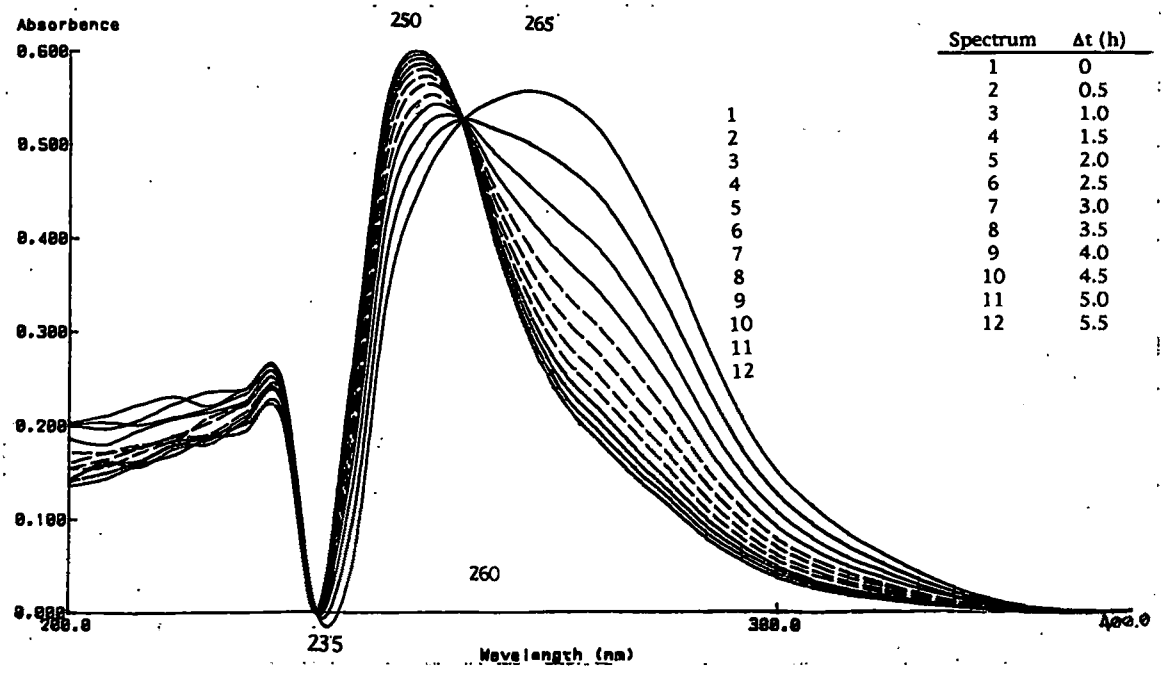


Figure 2.12 *UV/Vis spectra of hydrolysis of N-[4-[N',N'-bis(2-chloroethyl)amino]phenyl] acetamide* ***(11)*** *in aqueous DMSO (9:1,v/v) at 37°C and pH 2 :* $[11]_{init.}$ *ca.* $1x10^{-4}M$

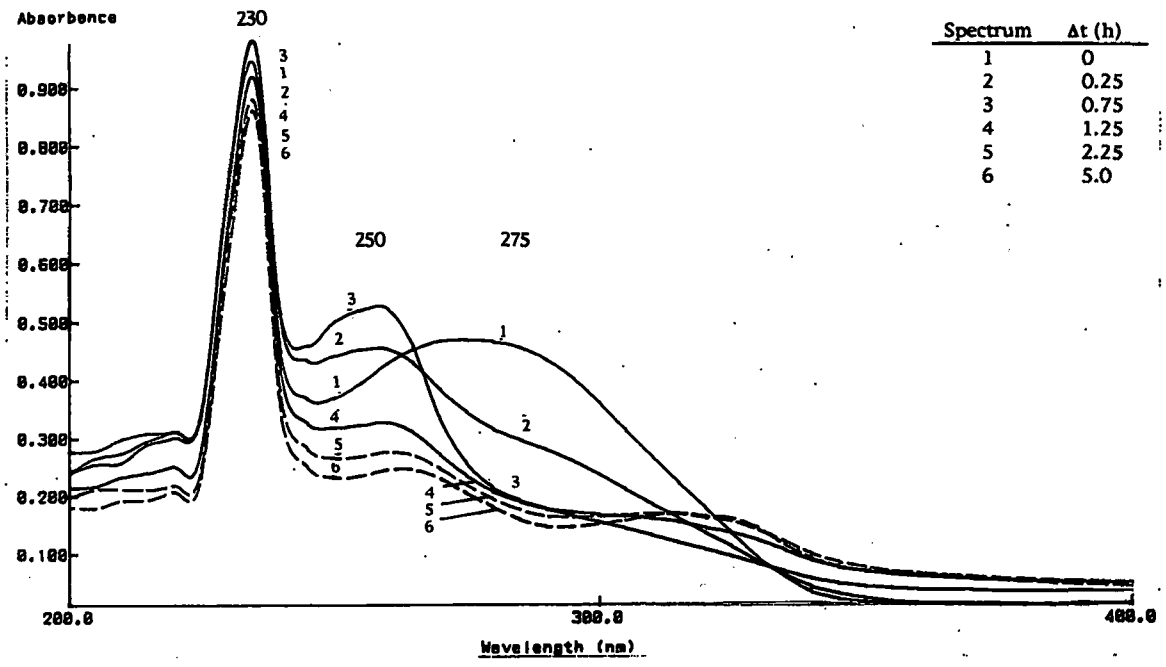


Figure 2.13 *UV/Vis spectra of hydrolysis of N-[4-N'N'-bis(2-chloroethyl)amino]phenyl] trifluoroacetamide (12) in aqueous DMSO (9:1,v/v) at 37°C and pH 12: [12]$_{init.}$ ca. 1x10^{-4}M*

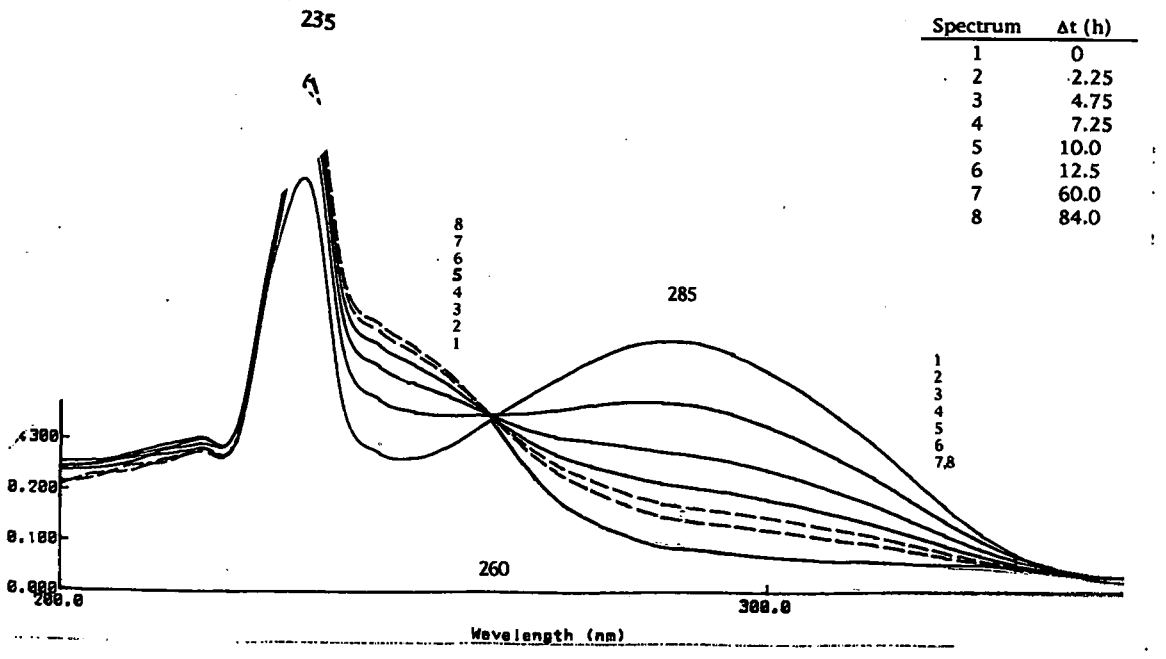


Figure 2.14 *UV/Vis spectra of hydrolysis of N-[4-N',N'-bis(2-chloroethyl)amino]phenyl] trifluoroacetamide (12) in aqueous DMSO (9:1,v/v) at 37°C and pH 2; [12]$_{init.}$ ca. 1x10^{-4}M*

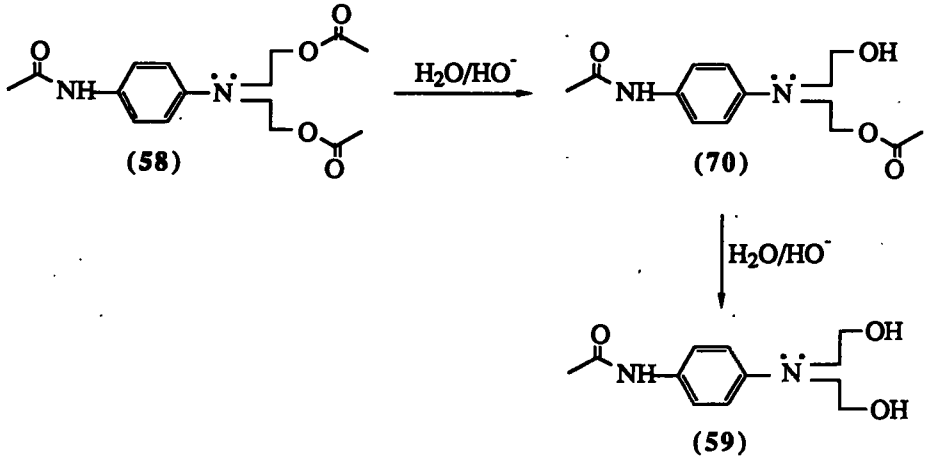
2.7 Stability of aryl mustard (58) in aqueous DMSO at 50°C and pH 12 determined by HPLC

N-[4-*N'*,*N'*-bis(2-acetyloxy)ethylamino]phenyacetamide **(58)** was synthesised to investigate the effect of nucleofugacity on the differential reactivity of aryl mustard prodrug/drug combinations. Unfortunately, lack of time prevented full examination of this factor.

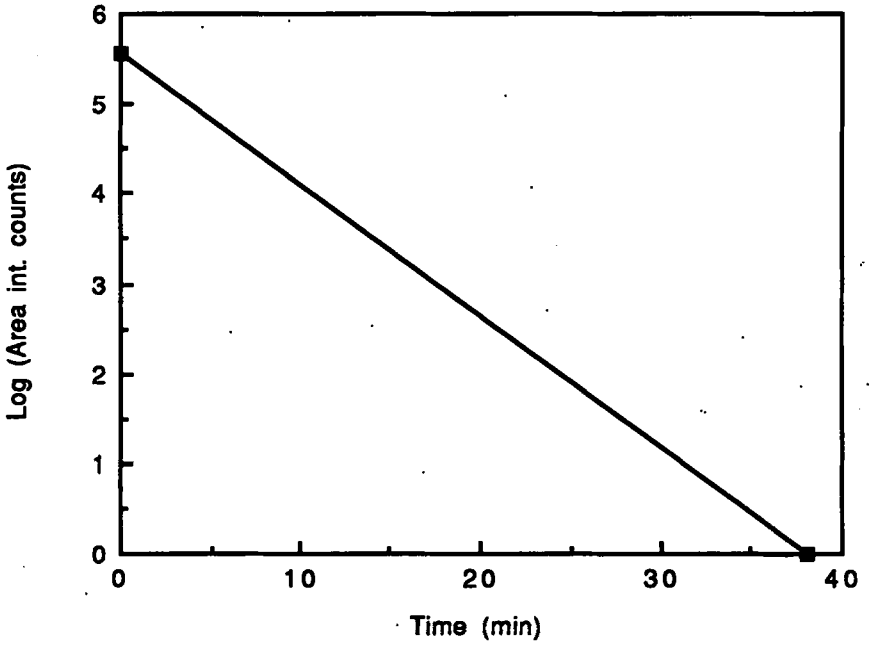
The stability of **(58)** was briefly examined in aqueous DMSO (7:3, v/v) at 50°C and pH 12 using reversed-phase HPLC to follow both the loss of **(58)** (Rf=19.91 min) and the formation of both the half-hydrolysed mustard **(70)** (Rf=15.16 min) and the final hydrolysis product 4-[*N*,*N*-bis(2-hydroxyethyl)]-phenylacetamide **(59)** (Rf=8.63 min) (*Scheme* 2.12) (Experimental 5.4.3.2 and 5.4.3.3). The identity of **(59)** was established by comparison of HPLC retention time against authentic compound and by mass spectral examination of the reaction product.

The *pseudo* first order rate (k_1) for decomposition of **(58)** in aqueous DMSO (7:3, v/v) at pH 12 and 50°C, determined from the plot of log (peak area) of **(58)** *versus* time, was *ca.* 2.4 x 10^{-3} s^{-1}, and the HPLC trace of the reaction mixture after 38 min showed no trace of **(59)**. Further, the intermediate **(70)** increased, passed through a maximum concentration at *ca.* 40 min and then disappeared (*Figure* 2.15).

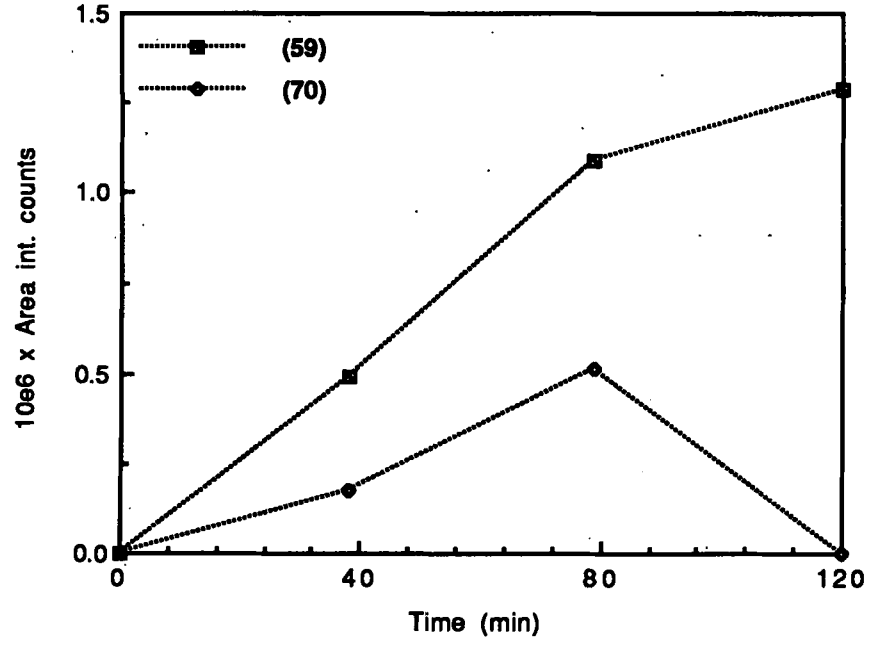
Other brief experiments (Experimental 5.4.3.3) showed **(58)** was very stable in aqueous phosphate buffer (3% v/v DMSO) at pH 7.4 and 37°C, showing only *ca.* 5% decomposition over 24h.



Scheme 2.12 *Decomposition pathway for N-[4-N',N'-bis(2-acetyloxyethyl)amino]phenyl-acetamide (58) in aqueous DMSO (7:3,v/v) at pH 12 and 50°C*



a) Decomposition of starting material (58), Rf=19.91 min



b) Formation of decomposition products: (59), Rf=8.63 min; (70), Rf=15.16 min

Figure 2.15 *Hydrolysis of N-[4-N',N'-bis(2-acetyloxy)ethyl]amino]phenyl]acetamide (58) in aqueous DMSO (7:3, v/v) pH 12 at 50°C: [58]* $_{init.}$ *ca. 1x10*$^{-4}$*M*

2.8 The common ion effect by added Cl^-

Retardation by added Cl^- of the hydrolysis of mustards (10), (11) and (12) was also briefly examined by UV-visible spectroscopy. The mustard in DMSO (10 μl, $1.5x10^{-2}$M) was injected into a solution (3 cm^3) of the salt (0.5M) (NaCl or $NaClO_4$) at pH 2 (adjusted with 1M HCl) in DMSO (9:1, v/v) contained in a cuvette thermostatted at 37°C. This gave an initial substrate concentration of *ca.* 5×10^{-5}M. The first spectrum was recorded immediately after addition of the substrate and then at timed intervals. Similar reactions were run in dilute HCl, and $HClO_4$. The results in ***Table 2.8*** show clearly that addition of NaCl retards the acid hydrolysis of (10), (11) and (12). The retardation is larger for NaCl than $NaClO_4$, which suggests the operation of a common ion rather than a salt effect. These results agree with previous observations[16,113] on the common ion effect observed for Cl^-.

Table 2.8

Half-lives ($t_{1/2}$) for the hydrolysis of aryl mustards (10), (11) and (12) in aqueous DMSO (9:1,v/v) at pH 2 and 37°C in the presence of NaCl or $NaClO_4$

Compound	$t_{1/2}$ (h)			
	0.01 M HCl	0.01 M HCl, 0.5M NaCl	0.01 M HCl, 0.5 M NaClO4	0.1M $HClO_4$
N,N-Bis(2-chloroethyl)--4-amino-aniline (10)	17	20	9	6.5
N-[4-[*N'*,*N*-Bis(2-chloroethyl) amino-phenyl]acetamide (11)	2	5	2	?
N-[4-[*N'*,*N*-Bis(2-chloroethyl)-amino]phenyl]trifluoroacetamide (12)	5	9	3.5	4.5

2.9 Synthesis of authentic products (59),(60),(65) and (66)

These were synthesised to facilitate product analysis for the hydrolysis of mustards (11) and (12). Both (60) and (65) are not described in the literature.

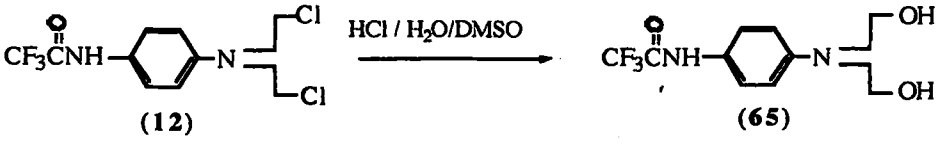
N-[4-[*N'*,*N'*-Bis(2-hydroxy)ethyl]amino]phenyl]acetamide (59) was obtained by mild, base hydrolysis of *N*-[4-[*N'*,*N*'-bis(2-acetyloxy)ethyl]amino]phenyl]acetamide (58) using aqueous NaOH (pH 12) at 37°C for 5h, (*Scheme 2.12*), a different route to the one

followed by DeGraw *et al.* [160] who react 4'-aminoacetanilide with ethylene oxide in aqueous acetic acid.

The reaction was followed by HPLC: the starting material **(58)** gave Rf=19.91 min and the final hydrolysis product **(59)** gave Rf=8.63 min; the formation of an intermediate compound **(70)** (Rf=15.16 min) (presumably from removal of only one of the two O-acetyl functions) was also apparent. The reaction solution was neutralised with gaseous CO_2 to pH 7 before extraction with DCM. Compound **(59)** was obtained in 87% yield and was not subjected to further purification. It showed m.p. 142-150°C (decomp.), (lit.[160] m.p. 141-142.5°C) and the MS(EI) spectrum gave m/z=238 corresponding to M^+. The ^{1}H-NMR spectrum in CD_3OD showed a singlet (3H) at 2.1 ppm for the acetyl group, two double doublets (8H in total) centred at 3.5 and 3.6 ppm with J=5 Hz for the hydroxyethyl CH_2 groups, an AB quartet (4H) at 6.7-7.4 ppm with J=10 Hz for the aromatic protons, and an exchangeable 3H singlet at 4.9 ppm for the OH and NH protons. The IR spectrum showed a strong absorption at 3500 and 1560 cm^{-1} corresponding to the alcohol and the carbonyl group, respectively.

Synthesis of 4-morpholinophenylacetamide **(60)** involved a simple acetylation with acetyl chloride of 4-morpholinoaniline in THF at room temperature for 1h. Triethylamine was added to neutralise released HCl. After purification by silica column chromatography using DCM as eluent, **(60)** was obtained as a white solid in 78% yield with m.p. 198-200°C. The MS(FAB$^+$) spectrum showed m/z=221 corresponding to MH^+ and MS(FAB$^-$) m/z=219 corresponding to $M\text{-}H^+$. The ^{1}H-NMR spectrum in $CDCl_3$ contained a singlet (3H) at 2.14 ppm for the methyl group; two sets of triplets (4H each) centred at 3.11 and 3.86 ppm (J=1.4 Hz) for the morpholine ring; an AB quartet (4H) at 6.88-7.38 ppm (J=3 Hz) for the aromatic protons and a broad exchangeable singlet (1H) at 7.41 ppm for the NH proton. The IR spectrum showed a strong absorption at 1660 cm^{-1} for the amide carbonyl group.

N-[4-*N*'*N*'-Bis-(2-hydroxyethyl)amino]phenyl]trifluoroacetamide **(65)** was obtained by acid hydrolysis (pH 2, HCl) of the mustard 4-[*N*',*N*'-bis(2-chloroethyl)amino] phenyl]trifluoroacetamide **(12)** in aqueous DMSO (7:3,v/v) for 12h at 37°C (*Scheme 2.13*).



(12) **(65)**

Scheme 2.13 Synthesis of (65)

The reaction was followed by HPLC and when all the starting material had been consumed, the mixture was neutralized (NaOH) and freeze dried. The residue was extracted into ethyl acetate. The product (89% yield) was not further purified as HPLC showed a single peak at Rf=17.75 min. The MS(FAB$^+$) spectrum showed m/z=293 corresponding to MH$^+$. The ^{1}H-NMR spectrum in $(CD_3)_2CO$ showed two sets of triplets (4H each) centred at 3.62 and 3.75 ppm with J=1.3 Hz for the hydroxyethyl CH_2 groups, and an AB quartet (4H) at 6.98-7.59 ppm with J=2 Hz for the aromatic protons. The IR spectrum contained strong absorptions at υ= 3500 (for the alcohol group) and 1700 cm^{-1} for the amide carbonyl.

N,N-Bis(2-hydroxyethyl)-4-aminoaniline **(66)** was obtained impure in 34% yield by neutralization of an ethanolic solution of *N,N*-bis(2-hydroxyethyl)-4-amino aniline sulphate salt (previously recrystallised from ethyl alcohol) with one equivalent of aqueous NaOH (1M). After 10 min, the solution was freeze-dried and the residue extracted into ethyl acetate. It proved impossible to purify and gave m.p. 85-95°C, (lit.[157] m.p. 87-88°C) The MS(FAB$^+$) spectrum showed m/z=197 corresponding to MH$^+$ and the ^{1}H-NMR spectrum in $(CD_3)_2SO$ {doublet (8H) at 3.2 ppm for the CH_2 groups, an AB quartet (4H) at 6.8-7.5 ppm for the aromatic protons, and a broad exchangeable singlet (2H) at 4.9 ppm for the NH or OH groups} is consistent with isolation of the neutral compound **(66)**.

Chapter 3

Results and Discussion 2: Carbamate Prodrugs

3.1 Utility of carbamate compounds as prodrugs

The most potent aryl mustards are those bearing electron donating substituents such as the 4-amino group, and *N,N*-bis(2-chloroethyl)-4-amino-aniline **(10)** appears to be one of the most reactive mustards studied.[119]

The aim of the work reported in this chapter was to prepare and evaluate model aryl prodrugs containing a 4-amino group deactivated by the carbamate moiety. This carbamate moiety was expected to decompose spontaneously to generate the 4-amino-aryl-mustard drug following activation by an enzyme, possibly the acylarylamidase E.C.3.5.1.13, used in previous experiments involving ADEPT (see Appendix). *Scheme 3.1* briefly summarises the sequence of steps involved. Action of the enzyme on the protected 4'-amino substituent terminal of carbamate **(71)** was envisaged to generate the reactive carbamate **(72)** bearing a free 4'-amino group. Electron donation by the 4'-amino group was anticipated to spontaneously cleave the carbamate function, with liberation of CO_2, to release the active 4-amino aryl compound **(73)**. If the group Z was the *N,N*-bis(2-chloroethyl)amine structure, this would generate the active mustard drug, *N,N*-bis(2-chloroethyl)-4-amino-aniline **(10)**.

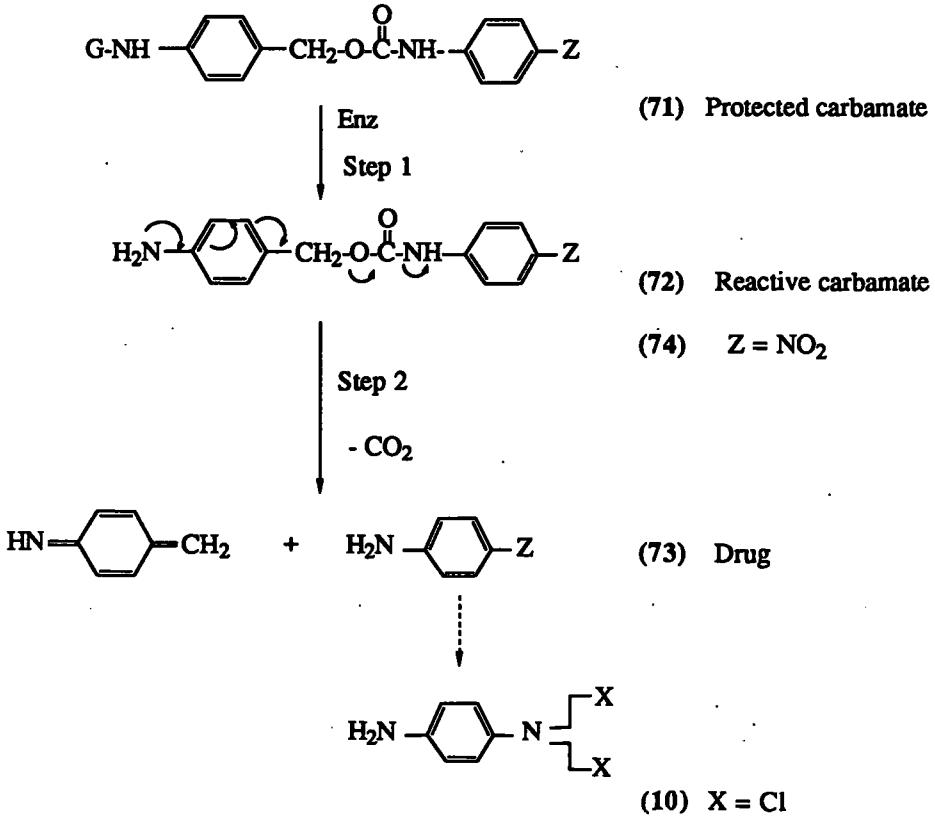
The work reported in the following pages concerns the synthesis of 4'-aminobenzyl *N*-(4-nitrophenyl) carbamate **(74)** (where Z=NO_2), and examination of its stability in organic and aqueous media at 37°C. Identification of the decomposition products gave some insight into the mechanism of decomposition. The nitro group was chosen because it can be easily converted into a mustard moiety to progress development of the prodrugs.

Synthesis of (74) was difficult due to the free 4'-amino group. It was generated from the corresponding protected 4'-amino carbamate (71) by chemical removal of the protecting group G. The best conditions for the removal of the protection were investigated.

Four precursors of (74) with different protecting groups G were investigated - benzyloxycarbonyl (CBZ) (75) and (76) (exception for Z=CF_3), triphenylmethyl (Trityl) (77), *t*-butoxycarbonyl (*t*-BOC) (78), and acetyl (Ac) (26) derivatives. Synthesis of these compounds, their stability, and the method of removal of the protection, are described and evaluated.

The N'-*t*-butoxycarbonyl protected carbamate (78) proved best for the synthesis of (74). Conditions were found for the removal of the N'-*t*-butoxycarbonyl group without damaging the central benzyloxycarbonyl bond in the molecule. Compound (74) was obtained in crystalline form as a picrate derivative.

The *N'*-acetyl protected carbamate (26) was synthesised, its stability evaluated and it was examined as a substrate for the acylarylamidase E.C. 3.5.1.13 enzyme. It was found to generate (74) and is therefore a potential prodrug.



(71) Protected carbamate

(72) Reactive carbamate

(74) Z = NO_2

(73) Drug

(10) X = Cl

Scheme 3.1 Mechanism for conversion of the carbamate prodrug into the active drug

3.2 Synthesis of 4'-amino protected carbamates and removal of the protection group

Many different groups have been used for the protection of the amino functions, dependent on the synthetic conditions.[161-165] A prime requirement for the 4'-amino function of compound **(74)** was removal of the protection under conditions that would not damage the central carbamate function. This function is base labile,[166] so its removal must be effected by either mild acid or hydrogenolyses.

Four different protection groups were examined: benzyloxycarbonyl (CBZ) and the related *t*-butyloxycarbonyl (*t*-BOC), both giving carbamate derivatives; triphenylmethyl (trityl), giving an N-alkyl derivative; and finally acetyl (Ac) to form an amide derivative.

According to the literature,[162,167,168] the CBZ group can be introduced by reacting aqueous solutions of the amino derivative with benzyl chloroformate, at slightly alkaline pH (e.g. using MgO), at temperatures between 0 and 20°C. Its removal can be achieved with good selectivity by catalytic hydrogenolysis.[167] Other methods of removal include reaction with trialkylsilanes,[169] acid hydrolysis,[167] photolytic cleavage[170] or electrochemical reduction.[171]

The trityl group can be introduced by reaction with triphenylmethyl chloride in the presence of mild base[172] and removed by very mild acid hydrolysis.[172] It can also be removed by catalytic hydrogenolysis,[173] but more slowly than the CBZ group.

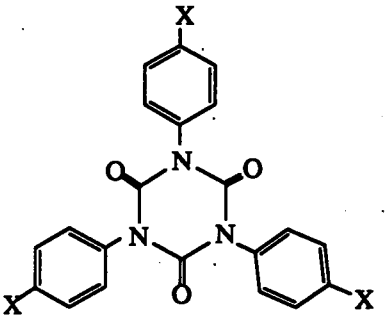
The *t*-BOC group can be introduced by reaction with di-*t*-butyl dicarbonate[174] or *t*-butyl azidoformate[175] at room temperature, and easily removed by very mild acidic hydrolysis using either trifluoroacetic acid [176] or formic acid.[177] Removal of *t*-BOC by HCl (3M) in ethyl acetate is also reported.[178] Recently, the selective removal of *t*-BOC in the presence of benzyl ester and benzyloxycarbonyl groups was achieved with mercaptoethane-sulphonic acid in glacial acetic acid.[179,180] *t*-BOC shows resistance towards hydrogenolysis and alkaline hydrolysis.[162]

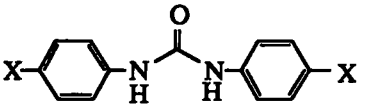
The Ac group can be introduced with a variety of acylating agents. It is difficult to remove chemically, but enzymes are effective under mild conditions.[181]

The synthesis of the N'-protected carbamates N'-CBZ **(75)** and **(76)**, N'-trityl **(77)**, N'-*t*-BOC **(78)** and N'-Ac **(26)** was achieved in two sequential steps generalised in *Scheme 3.2.* The first step involves N-protection of 4-aminobenzyl alcohol **(80)**. The second

step is the condensation of the protected alcohol with an arylisocyanate. The addition of arylisocyanate is normally at 0°C followed by temperature increase to 25- 40°C.

For the N'-trityl and N'-*t*-BOC derivatives, this condensation proved difficult. The 4-nitrophenylisocyanate reacted with the protected alcohol slowly, or not at all, to give the trimer **(81)** and urea **(82)** as major products. Similarly, for reaction of protected alcohol with phenylisocyanate, there was a relatively large yield of trimer **(83)** and urea **(84)** derivatives. All the ureas were insoluble and they were identified by MS(EI) showing a strong peak corresponding to the M^+ ion at m/z = 492 for **(81)**, m/z = 357 for **(83)**, m/z = 256 for **(82)**, and m/z = 212 for **(84)**. To overcome this problem, both N-trityl and N-*t*-BOC protected alcohol was converted *in situ* into a more reactive anion, by reaction with n-butyllithium, just before the addition of arylisocyanate. Further, the arylisocyanate was added in four aliquots to avoid high concentrations favourable to self condensation.





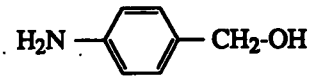
(81) X = NO_2 **(82)** X = NO_2

(83) X = H **(84)** X = H

Chart 10 *Structures (81) - (84)*

The *N'*-protected carbamate products **(26)**, **(75)**, **(76)**, **(77)** and **(78)** were purified by silica column chromatography, sometimes followed by recrystallisation . The low solubility of all the protected carbamates in most organic solvents complicated purification and contributed to the very low yields of recovered pure products.

The protected carbamates were characterized by their MS and ^{1}H-NMR spectra. The IR spectra confirmed the presence of the most significant functional groups: the secondary carbonyl carbamate shows a strong absortion at 1722-1705 cm^{-1} that agrees well with Pinchas' observations.[182] For some compounds, elemental analysis was realised to confirm the molecular formula.



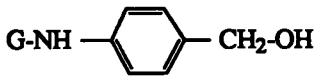
(80)

A. $C_6H_5CH_2O(CO)Cl$ / MgO,H_2O,Ether

B. $(C_6H_5)_3CCl$ / Et_3N, DCM

C. $O[CO_2C(CH_3)_3]_2$ / THF

D. 1) $O(COCH_3)_2$ / Py → $AcNH\text{-}C_6H_4\text{-}CH_2\text{-}OAc$ (95)
2.1) aq NaOH,EtOH 2.2) CO_2



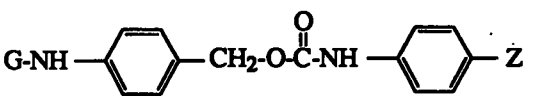
G = $(CO)OCH_2C_6H_5$	(CBZ)	A	(85)
G = $C(C_6H_5)_3$	(trityl)	B	(91)
G = $(CO)OC(CH_3)_3$	(*t*-BOC)	C	(93)
G = $(CO)CH_3$	(Ac)	D	(96)

A_1. $NO_2\text{-}C_6H_4\text{-}NCO$ / ACN,DCM
A_2. $CF_3\text{-}C_6H_4\text{-}NCO$ / ACN,DCM

B. 1) n-BuLi / THF
2) $NO_2\text{-}C_6H_4\text{-}NCO$

C. 1) n-BuLi / THF
2) $NO_2\text{-}C_6H_4\text{-}NCO$

D. $NO_2\text{-}C_6H_4\text{-}NCO$ / ACN,DCM



G = $(CO)OCH_2C_6H_5$,	Z=NO_2	A_1	(75)
G = $(CO)OCH_2C_6H_5$,	Z=CF_3	A_2	(76)
G = $C(C_6H_5)_3$,	Z=NO_2	B	(77)
G = $(CO)OC(CH_3)_3$,	Z=NO_2	C	(78)
G = $(CO)CH_3$,	Z=NO_2	D	(26)

Scheme 3.2 Synthesis of the protected carbamates (26) and (75) - (78)

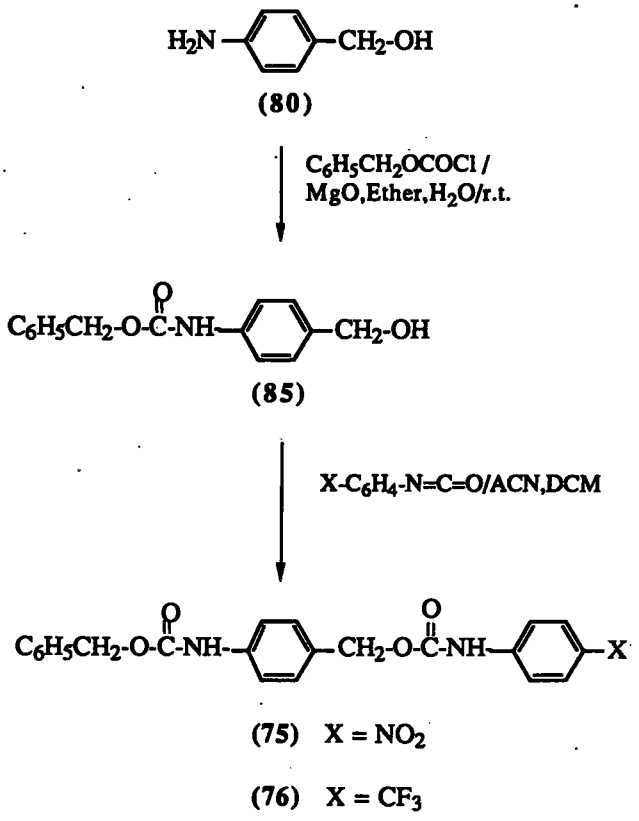
3.2.1 The *N*'-benzyloxycarbonyl derivatives (75) and (76)

3.2.1.1 Synthesis

The reactions for the synthesis of 4'-(*N*'-benzyloxycarbonylamino)benzyl *N*-(4-nitrophenyl) carbamate (75) and 4'-(*N*'-benzyloxycarbonylamino)benzyl *N*-(4-trifluoro-methylphenyl) carbamate (76) is shown in ***Scheme 3.3***. Both involved a similar sequence: (1), protection of the amine substituent of the 4-aminobenzyl alcohol (80) by conversion into a *N*-benzyloxycarbonyl derivative (85); and (2), condensation of this protected compound with 4-nitrophenylisocyanate to give (75) or with 4-trifluoromethylphenyl isocyanate to give (76).

The carbamates (75), (76) and (85) are new compounds.

4-(*N*-Benzyloxycarbonylamino)benzyl alcohol (85) was best synthesised by reaction of the 4-aminobenzyl alcohol with benzyl chloroformate, at room temperature, using MgO as the base catalyst, in a biphasic aqueous ether solvent (85% crude yield). Other bases (Et_3N,



Scheme 3.3 Synthesis of compounds (75) and (76)

Na_2CO_3) in acetonitrile or THF gave much lower amounts (10% crude yield). The reaction was monitored by silica tlc and the product purified by silica column chromatography using ether eluent. It was further purified by recrystallisation from ether/n-hexane (5:1,v/v) to give the pure **(85)** in 64% yield, with a sharp m.p. 94-95°C. The compound was characterised spectroscopically and detailed results are given in the Experimental (Section 5.3.22). The most significant findings were a peak at m/z=257 in the MS(EI) spectrum corresponding to $M^{+\cdot}$, and a strong IR absorption at υ=3300 cm^{-1} characteristic of the alcohol group and υ=1710 cm^{-1} corresponding to the carbamate carbonyl group.

4'-(*N*'-Benzyloxycarbonylamino)benzyl *N*-(4-nitrophenyl) carbamate **(75)** was obtained in a crude yield of 69% and after silica column chromatography using ether, followed by recrystallization from petroleum ether (40-60°C) in a pure yield of 20%, with m.p.159-160°C and a satisfactory elemental analysis. It was characterized spectroscopically. Thus, MS(FAB$^-$) shows a peak at m/z=420 due to M-H$^+$; ^{1}H-NMR in $CDCl_3$ shows a double singlet (4H) centred at δ=5.25 and 5.30 ppm assigned to the two CH_2 groups, a multiplet (9H) centred at 7.3 ppm due to the aromatic protons of the protecting group and the 4-amino benzyl moiety, and an AB quartet (4H) centred at 7.85 ppm (J=10.3 Hz) characteristic of the 4-nitroaniline moiety; the IR spectrum shows a strong absorption at 1730 cm^{-1} due to the carbamate functions, and no absorption around 3300 cm^{-1}, characteristic of the alcohol group.

The main impurities appeared to be the urea derivative $0_2N\text{-}C_6H_4\text{-}NH)_2CO$ [MS(EI) m/z=302; ^{1}H-NMR ($(CD_3)_2SO$) δ=8.1 ppm (8H, ABqu, J=10Hz)] and 4-nitroaniline [MS(EI) m/z=138; ^{1}H-NMR ($CDCl_3$) δ=7.5 ppm (4H, ABqu, J=10 Hz)].

4'-(*N*'-Benzyloxycarbonylamino)benzyl*N*-(4-trifluoromethylphenyl) carbamate **(76)** was obtained in a pure yield of 43%, after silica column chromatography (see Experimental) and gave m.p. 167-170.5°C. It was also characterised spectroscopically. Thus, MS(EI) shows m/z=444 due to M$^+$; ^{1}H-NMR ($CDCl_3$/$(CD_3)_2CO$) shows two singlets (2H each) at δ=5.1 and 5.15 ppm, due to the CH_2 groups of the benzyl and carbamate groups, plus a multiplet (13H) centred at 7.5 ppm due to the aromatic protons; the IR spectrum shows a strong absorption at 1720 cm^{-1} due to the carbamate carbonyl group.

3.2.1.2 Hydrogenolysis

Removal of the benzyloxycarbonyl protection from compounds **(75)** and **(76)** was effected by hydrogenolysis over Pd/C (10%), following a literature method.[167]

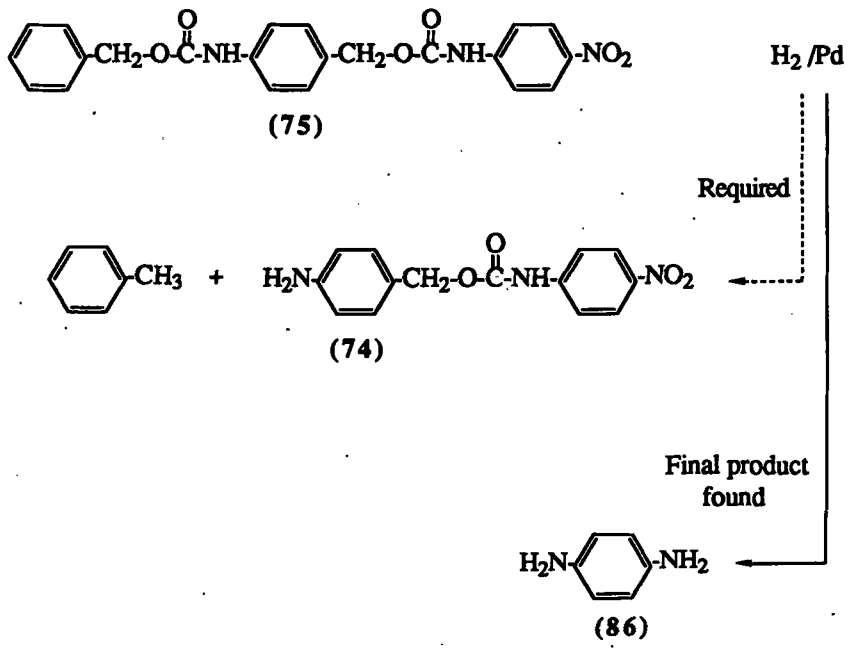
Both compounds gave unsatisfactory results insofar as the required deprotected carbamate was not obtained. Thus, **(75)** gave 1,4-phenylenediamine **(86)** rather than **(74)**

(*Scheme* 3.4), and (76) gave 4-aminobenzotrifluoride (87) rather than (88) (*Scheme* 3.6). Nonetheless, these reactions were examined further to investigate the sequence of benzyloxycarbonyl group cleavage.

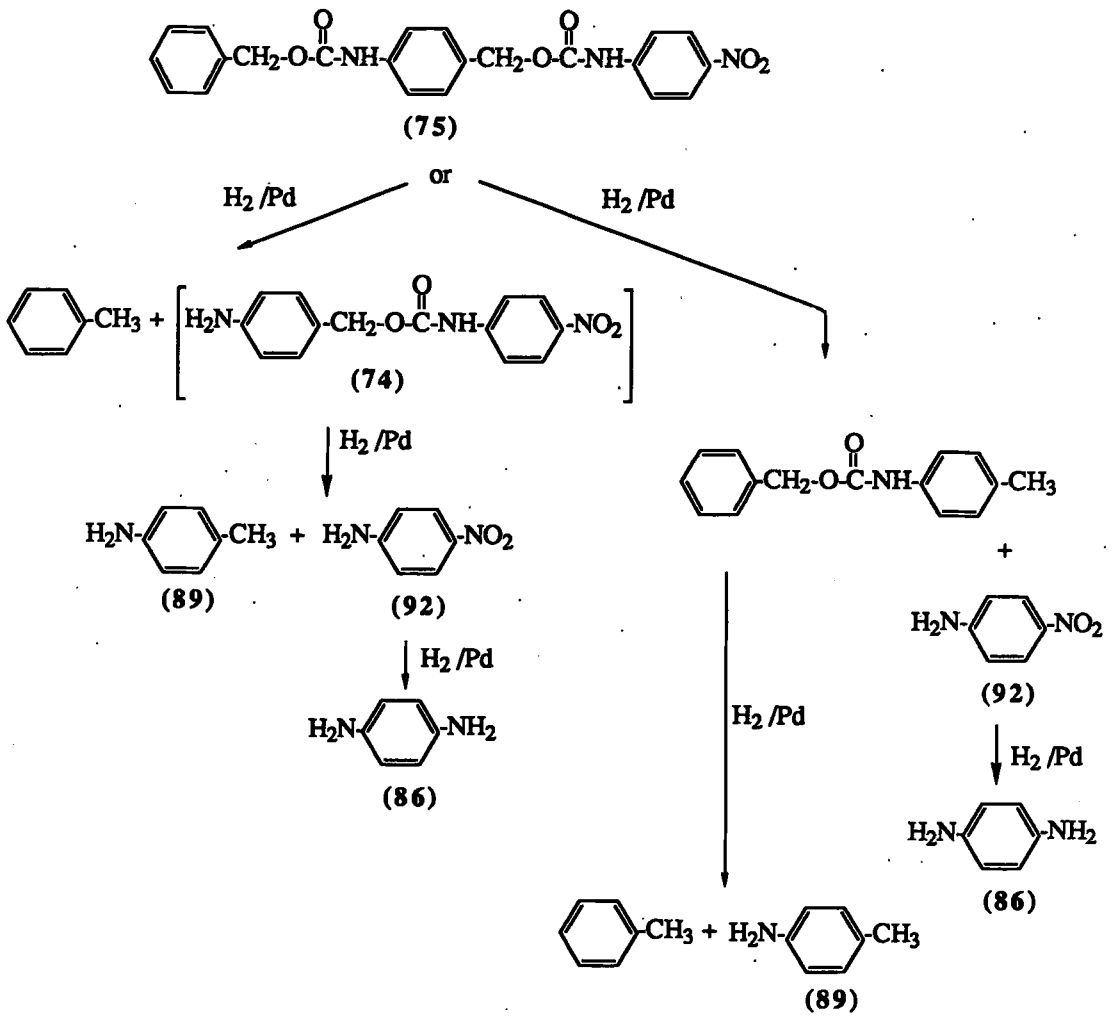
Hydrogenolysis of carbamate (75) (0.5 mmole) in ethyl acetate with Pd/C catalyst at room temperature was monitored by silica tlc against authentic of 4-toluidine (89) and 4-aminobenzyl alcohol (80). After 2h and 5h, the tlc showed the presence of 4-toluidine plus starting material (75). After 14h, neither 4-toluidine nor (75) were evident and a new diffuse spot had appeared on the tlc plate. As the hydrogen up-take corresponded to 1.5 mmol (i.e. 3-fold more than expected), the reaction was worked up to reveal the formation of 1,4-phenylenediamine (86) in 45% yield in the isolated reaction products [(MS(EI) m/z=108, M^+); ^{1}H-NMR ($CDCl_3$) δ=3.15 (4H, s, exch) and 6.5 ppm (4H,m)].

These results do not exclude the initial formation of 4-toluidine as evidence of deprotection of (75) and decomposition of (74). The absence of 4-toluidine among the final products may relate to its high volatility, and resulting loss, during hydrogenolysis and work-up.

Formation of 1,4-phenylenediamine requires not only removal of the N'-CBZ protection from (75), but also cleavage of the central urethane moicty and reduction of the 4-nitroaniline to 1,4-phenylenediamine, as outlined in *Scheme 3.5.*



Scheme 3.4 Hydrogenolysis of the N'-benzyloxycarbonyl derivative (75) for 14h



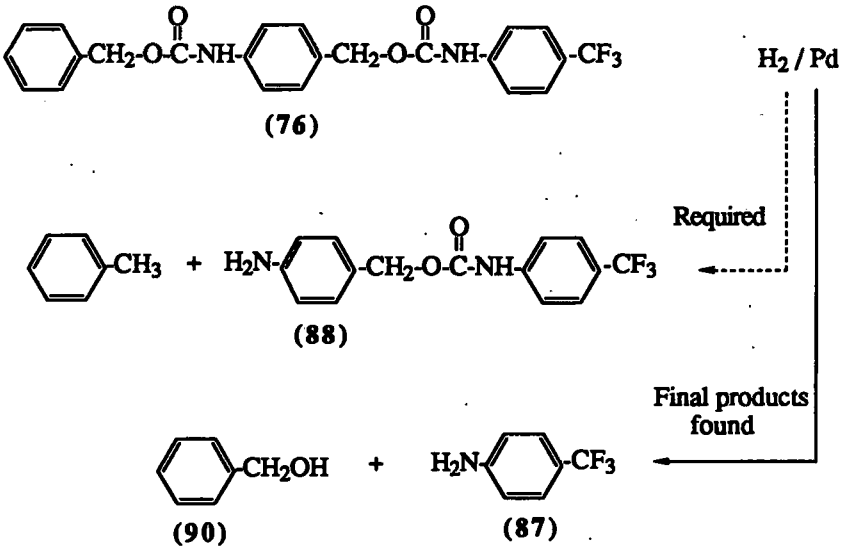
Scheme 3.5 Hydrogenolysis mechanism of N'-benzyloxycarbonyl derivative (75)

Because the hydrogenolysis of compound (75) was complicated by concurrent reduction of the 4-nitro group, an analogous compound (76), bearing the 4-trifluoromethyl group, was synthesised and examined.

On hydrogenolysis after 14h, (76) gave as final products, a mixture of 4-aminobenzotrifluoride (87) and benzylalcohol (90), rather than (88) (*Scheme 3.6*). Monitoring the reaction by GC using a BP20 column against authentic 4-aminobenzotrifluoride (87), benzylalcohol (90) and 4-(*N*-benzyloxycarbonylamino)benzyl alcohol (85), gave additional information about the sequence of product formation.

Hydrogenolysis of carbamate (76) (0.5 mmol) in ethanol with Pd/C catalyst at room temperature gave after 15 min, two GC peaks with the same retention times as 4-aminobenzotrifluoride (87) (Rf=13.91 min) and carbamate (85) (Rf=18.11 min). With time,

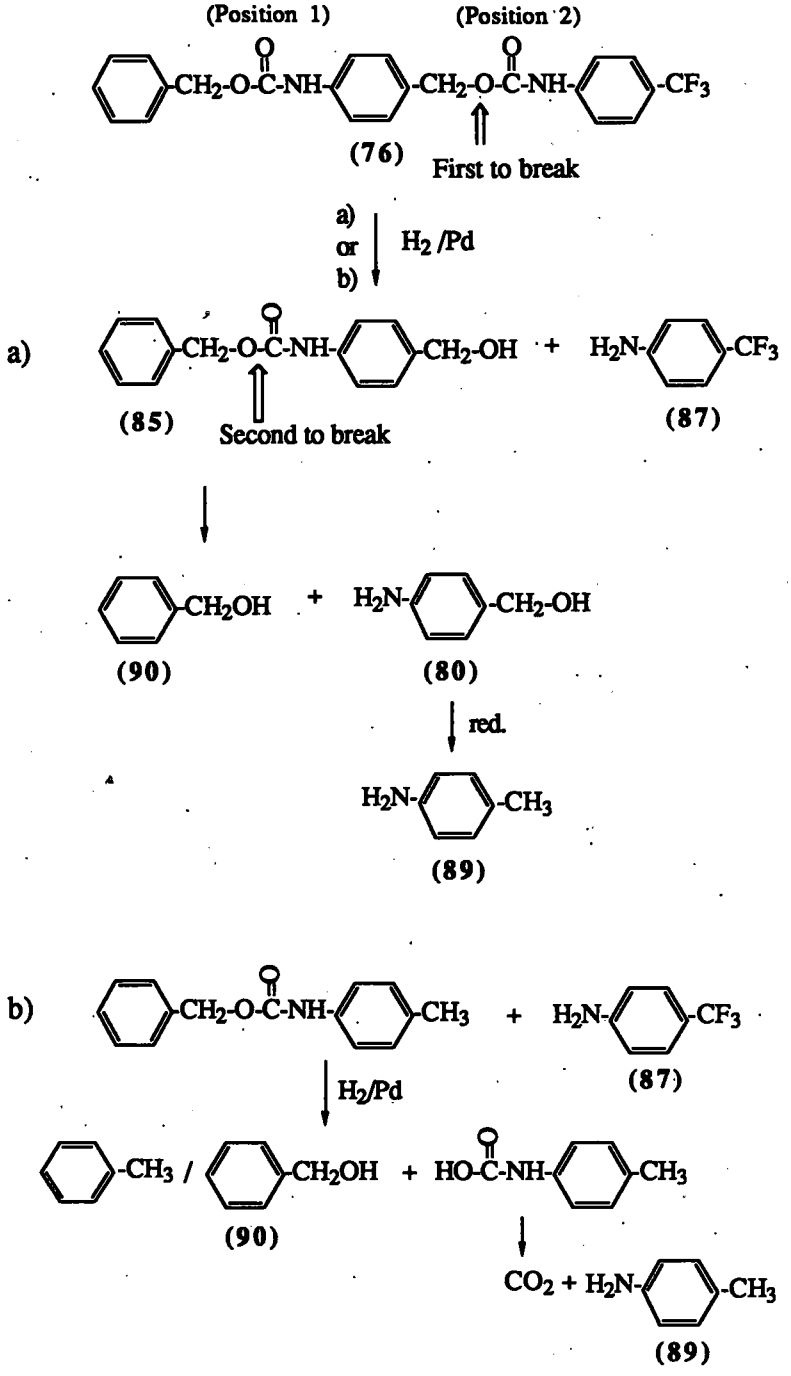
the area of the first peak increased to a constant value but the second peak disappeared and was not detected in further chromatograms. After 30 min, a third peak with the same retention time as benzylalcohol (90) (Rf=12.30 min) appeared, which increased with time to reach a maximum, and then decreased. Spiking with authentic compounds confirmed the identification of these three peaks as 4-aminobenzotrifluoride, carbamate (85) and benzylalcohol, respectively.



Scheme 3.6 Hydrogenolysis of the N-benzyloxycarbonyl derivative (76) for 14h

GC of samples taken after 2h showed the presence of new peaks which, along with some intermediates, were identified by GC-MS. These included 4-toluidine (89) (m/z=107), $H(CO)NHC_6H_4CH_3$ (m/z=135), possibly $H(CO)NHC_6H_4CH_2O(CO)H$ (m/z=151), an undentified compound with m/z=241, and the two previous observed 4-aminobenzotrifluoride (87) (m/z=161) and benzylalcohol (90) (m/z=108).

The results showing that compound (85) and 4-aminobenzotrifluoride (87) are the first detectable products on hydrogenolysis suggest that cleavage of the central carbamate moiety proceeds more rapidly than removal of the terminal N'-carbamate protection, as summarised by *Scheme 3.7*. Thus, the N-benzyloxycarbonyl group is inappropriate for protection of the terminal N-atom because hydrogenolysis preferentially removes the central carbamate moiety and it is therefore impossible to obtain the required prodrug.



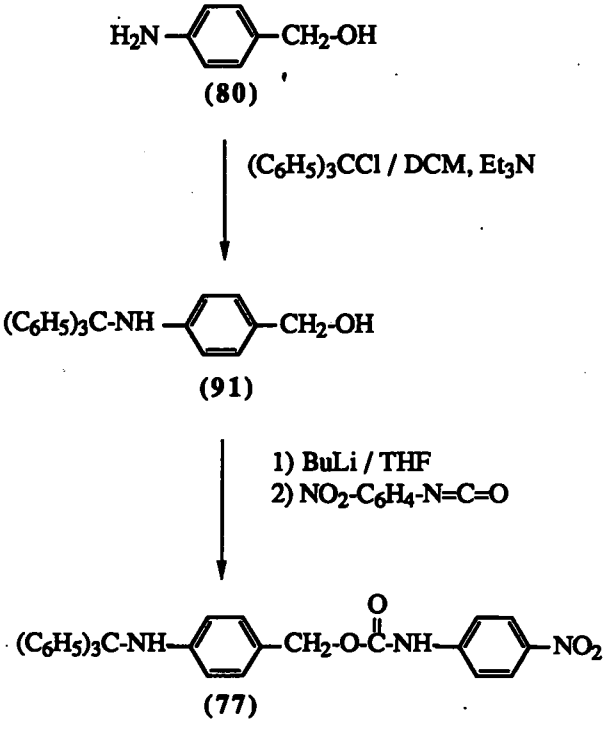
Scheme 3.7 Hydrogenolysis mechanism of carbamate (76)

3.2.2 The N'-triphenylmethyl derivative (77)

3.2.2.1 Synthesis

The synthesis of 4'-(*N*'-triphenylmethylamino)benzyl *N*-(4-trifluoromethylphenyl) carbamate (77) was effected by the reactions outlined in *Scheme 3.8.* These involved:

(1) reaction of 4-aminobenzyl alcohol **(80)** with triphenylmethyl chloride in DCM with one equivalent of triethylamine at 35°C to give quantitatively the crude trityl protected alcohol **(91)**. Purification by silica column chromatography followed by recrystallisation from DCM/n-hexane gave **(91)** in 77% yield; (2) condensation of **(91)** with 4-nitrophenyl isocyanate in the presence of one equivalent of n-butyllithium in THF to give the protected carbamate **(77)** in 76% yield after purification by silica column chromatography.



Scheme 3.8 Synthesis of compound (77)

4-(*N*-Triphenylmethylamino) benzyl alcohol **(91)** is a new compound. It gave m.p. 188-190°C and was identified spectroscopically. The MS(EI) spectrum showed the M^+ at m/z=365 and a base peak at m/z=243 due to the Ph_3C^+ (trityl) fragment. The ^{1}H-NMR spectrum in ($(CD_3)_2SO$) showed two exchangeable (1H) peaks at δ=3.4 (s) and 4.75 (t) ppm, due to the NH and OH protons, a 2H doublet at 4.2 ppm due to the CH_2 group and in the aromatic region, two sets of resonances at 6.55 ppm (4H, ABqu, J=8.7 Hz) and 7.25 ppm (15H, m). The IR spectrum confirms the presence of an alcohol group with an absorption at 3620 cm^{-1} and a secondary amine at 3440 cm^{-1}. The strong intensity of this peak suggests intramolecular association between the OH and NH groups.

4'-(*N*'-Triphenylmethylamino)benzyl *N*-(4-nitrophenyl) carbamate **(77)** gives m.p. 79-88°C and is unlikely to be pure. Silica tlc of **(77)** shows the presence of a trace impurity that proved impossible to remove by column chromatography. The elemental analysis shows that the carbon content is higher than expected (found: 76.7%, calculated: 74.8) with the nitrogen content slightly less (found: 6.9%, calculated: 7.9). The high carbon content impurity may well be a trityl compound like Ph_3COH. The MS(EI) spectrum shows no peak corresponding to M^+, instead it shows a peak at m/z=485 corresponding to $M^+ - CO_2$, and a base peak at m/z=243 due to the Ph_3C^+ fragment. The ^{1}H-NMR spectrum in $CDCl_3$ is consistent with the structure and impurity conclusions. It shows a singlet (2H) centred at δ=4.4 ppm due to the CH_2 group, two sets of AB quartets (J=2.0 and 2.2 Hz) that integrate to 4H each and are centred at 6.5 and 6.8 ppm, respectively, and a multiplet centred at 7.3 ppm for the trityl group that integrates to 17H instead of 15H. Finally, the IR spectrum shows a strong absorption at 1730 cm^{-1} due to the carbamate carbonyl function.

3.2.2.2 Hydrolysis

The problem with the use of a triphenylmethyl group as protection lies in the extreme difficulty of obtaining a pure compound. Purification by silica column chromato graphy generates an impurity probably due to Ph_3COH, and recrystallisation from ether/ n-hexane did not remove it. This behaviour is due to the extreme lability of the triphenylmethyl group.

In order to evaluate the lability of the triphenylmethyl group, carbamate **(77)** was hydrolysed in aqueous acetone (1:1,v/v, pH 2) at 25°C over 2h, and the reaction was followed by silica tlc against 4-nitroaniline **(92)**, 4-aminobenzyl alcohol **(80)** and 4-(*N*-triphenylmethylamino)benzyl alcohol **(91)**. It was possible to observe after 2h, the complete disappearance of **(77)** and among several final products only 4-nitroaniline was identified.

Similarly, the *N*'-triphenylmethyl alcohol **(91)** was hydrolysed under the same conditions. A tlc after 2h showed complete disappearance of **(91)** and no evidence for the presence of 4-aminobenzyl alcohol.

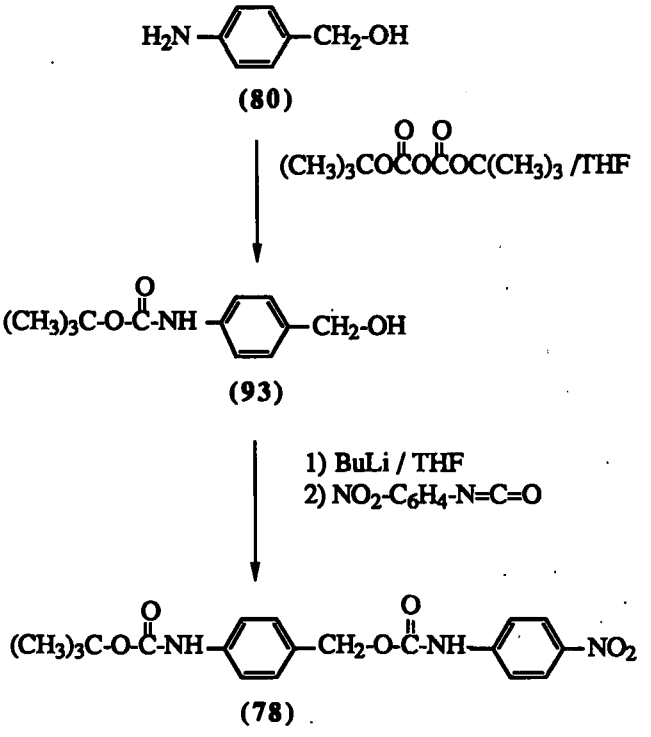
3.2.3 The N'-*tert*-butoxycarbonyl derivative (78)

3.2.3.1 Synthesis

The synthesis of 4'-(*N*'-*tert*-butoxycarbonylamino)benzyl *N*-(4-nitrophenyl) carbamate **(78)** was effected by the sequence of steps in *Scheme 3.9*. The reactions are as follows: (1) formation of the N-BOC derivative of 4-aminobenzyl alcohol **(93)** by reaction with

di-*t*-butyl dicarbonate in THF; (2) condensation of the protected alcohol (93) with 4-nitrophenyl isocyanate to give the *N'*-protected carbamate (78); this reaction was carried out in two steps, first adding *t*-butylithium in THF to generate the alcohol anion which was immediately reacted with the isocyanate.

Both (93) and (78) are new compounds.



Scheme 3.9 Synthesis of compound (78)

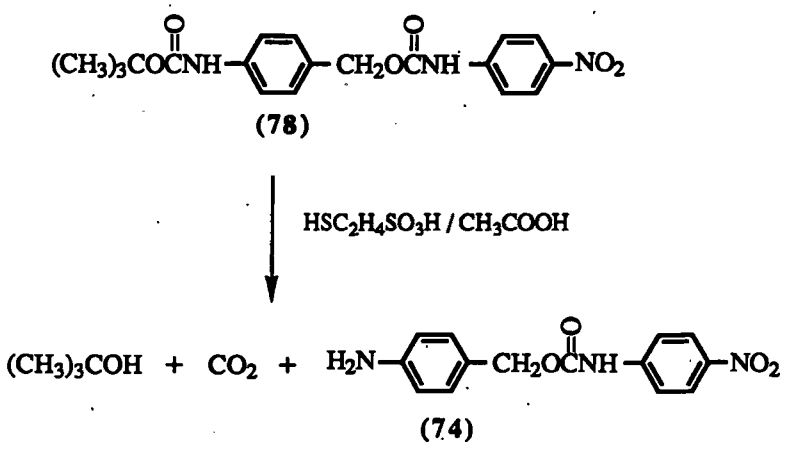
The synthesis of 4-(*N-tert*-butoxycarbonylamino)benzyl alcohol (93) followed a literature procedure.[174] After purification by silica column chromatography, pure (93) was obtained in 75% yield and gave m.p. 79-81°C and a satisfactory elemental analysis. MS(EI) showed m/z=223 corresponding to M^+ and a base peak at m/z=57 corresponding to the fragment $^+C(CH_3)_3$. The ^{1}H-NMR ($CDCl_3$) showed a singlet (9H) at δ=1.48 ppm due to $C(CH_3)_3$ group, a second singlet (2H) at 4.47 ppm attributed to the CH_2 group and an AB quartet (4H, J=2.2 Hz) centred at 7.15 ppm for the aromatic protons. Two broad exchangeable ^{1}H singlets, at δ=3.77 and 7.29 ppm relate to the OH and NH groups. The IR spectrum showed strong absorptions at 3480, 3380 and 1700 cm^{-1} attributed to the OH, NH, and carbamate carbonyl groups, respectively.

Pure 4'-(*N'-tert*-butoxycarbonylamino)benzyl *N*-(4-nitrophenyl) carbamate **(78)** was obtained in 61% yield after silica column chromatography followed by recrystallisation from ether/n-hexane: it gave m.p. 158-161°C, and a satisfactory elemental analysis. The MS(EI) spectrum showed m/z=387 corresponding to M^+ and a base peak at m/z=57 from the $^+C(CH_3)_3$ fragment. ^{1}H-NMR ($CDCl_3$) showed a singlet (9H) at δ=1.52 ppm for the $C(CH_3)_3$ group, a second singlet (2H) at 5.15 ppm, attributed to the CH_2 group, two AB quartets (both 4H and J=2 Hz) centred at 7.35 and 7.76 ppm for the aromatic protons, plus two exchangeable singlets at 6.55 and 7.17 ppm attributed to the NH protons. The IR spectrum confirmed the presence of two carbamate carbonyl groups showing a strong absorption at 1760 and 1715 cm^{-1}.

3.2.3.2 Hydrolysis of (78) and synthesis of picrate (94)

On treatment with HCl (3M) in ethyl acetate, followed by neutralization (5% aqueous $NaHCO_3$) and extraction into DCM, **(78)** gave several products, as shown by silica tlc. The MS(EI) m/z=287 confirmed the presence of **(74)**, but the yield of less than 10% was unsatisfactory.

Hydrolysis of **(78)** with mercaptoethanesulfonic acid in 20% (v/v) aqueous acetic acid at 25°C was more successful (***Scheme 3.10***). Silica tlc of the reaction mixture prior to work-up showed the presence of **(74)** in high yields (> 50%). Neutralization of the mixture with aqueous sodium bicarbonate developed a deep yellow colour and a silica tlc showed extensive formation of 4-nitroaniline **(92)**. The hydrolysis product **(74)** was therefore isolated from the acidic reaction mixture by conversion to the picrate derivative **(94)**, by adding aqueous picric acid (1%, w/w), and cooling to 0°C, in a yield of 84% (***Scheme 3.13***). The MS(FAB^+) spectrum of the picrate showed m/z=289 due to $NO_2C_6H_4NH(CO)OCH_2C_6H_4NH_3^+$, and the MS($FAB^-$) spectrum showed m/z=228 due to the picrate anion.



Scheme 3.10 Hydrolysis of compound (78)

3.2.3.3 Study of the stability of (78) in mild basic conditions

In view of the problems encountered in removing the N-BOC protection from (78), especially rapid decomposition during the basic work-up, the stability of (78) in THF containing saturated aqueous sodium bicarbonate was examined at 25°C over 24h. Both MS and ^{1}H-NMR assays of extracts of the reaction mixture showed more than 90% of the starting material (78) remained. This implies that (78) is relatively stable in mild base, and the low product yields on neutralising acidic reaction solutions with 5% aqueous sodium bicarbonate relate to the instability of (74) rather than (78).

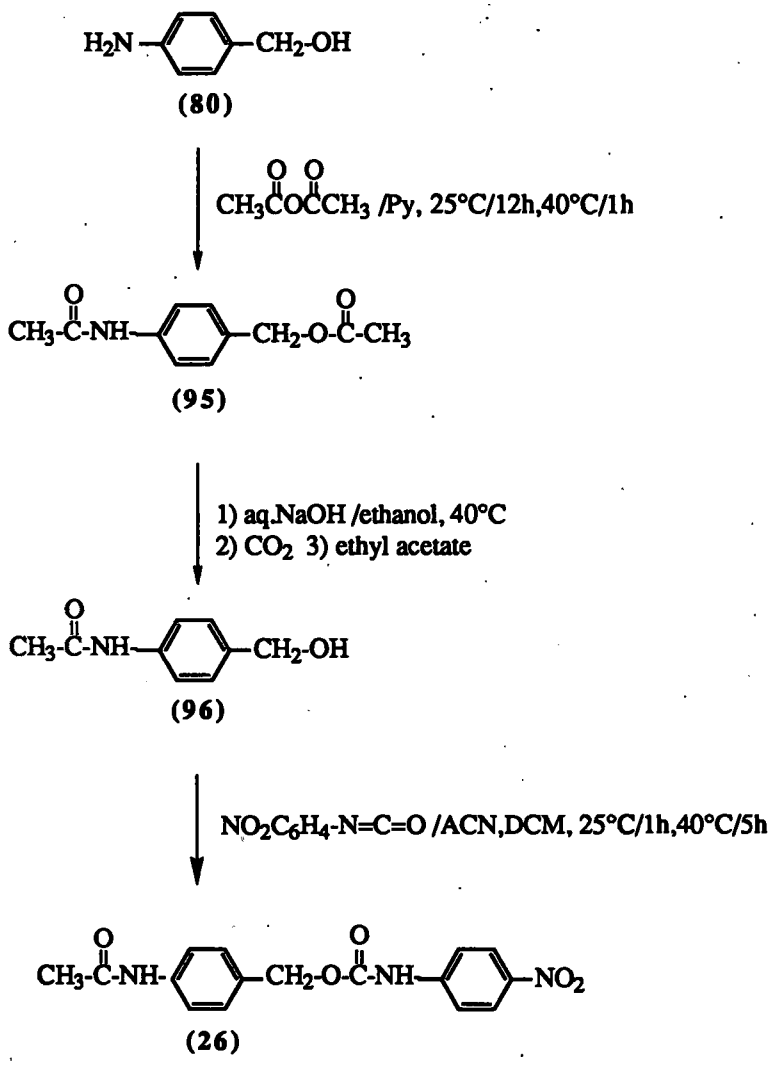
3.2.4 The N'-acetyl derivative (26)

3.2.4.1 Synthesis

The synthesis of 4'-(*N*'-acetylamino)benzyl *N*-(4-Nitrophenyl) carbamate (26) involved the series of reactions outlined in *Scheme 3.11*. These are: (1) Reaction of 4-aminobenzyl alcohol with acetic anhydride in pyridine at 25°C for 12h and a further 1h at 40°C, to give 4-(*N*-acetylamino)benzyl methyl ester (95). (2) Mild, basic hydrolysis at 40°C to selectively remove the ester function to give 4-(N-acetylamino)benzyl alcohol (96). (3) Condensation of (96) with 4-nitrophenylisocyanate in acetonitrile and DCM at 25°C for 4h and a further 5h at 40°C. Carbamates (26) and (96) are new compounds.

4-(*N*-Acetylamino)benzyl methyl ester (95) was obtained in a crude yield of 93% (m.p. 101-103°C) and it was used in the subsequent reaction without purification. The MS(EI) showed m/z=207 corresponding to M^+; ^{1}H-NMR ($(CD_3)_2CO$) showed two singlets (3H) at δ=2.02 and 2.07 ppm attributed to $OCOCH_3$ and $NHCOCH_3$, respectively, a third singlet (2H) at 5.01 ppm due to the CH_2 group, an AB quartet (4H) centred at 7.51 ppm due to the aromatic protons and a broad, exchangeable singlet (1H) at 9.21 ppm, attributed to the NH proton; the IR spectrum showed strong absorptions at 1760 and 1680 cm^{-1} corresponding to the ester and amide carbonyl functions.

4-(*N*-Acetylamino)benzyl alcohol (96) was obtained in 76% yield from hydrolysis of (95) in aqueous-ethanolic NaOH at pH 12 and at 40°C for 6h, followed by neutralization with CO_2 and extraction into ethyl acetate. It gave m.p. 115-117.5°C; MS(EI) gave m/z=165 corresponding to M^+; the ^{1}H-NMR spectrum ($(CD_3)_2CO$) showed a singlet (3H) at δ=2.1 ppm due to the N-acetyl group, a doublet (2H, J=7 Hz) centred at 4.6 ppm attributed to the CH_2 group, an AB quartet (4H, J=11 Hz) centred at 7.45 ppm for the aromatic protons, and two



Scheme 3.11 Synthesis of the N'-acetyl derivative (26)

exchangeable peaks (a triplet,1H, J=7 Hz, centred at 4.2 ppm and a broad singlet (1H) at 9.1 ppm, due to OH and NH, respectively); the IR spectrum showed strong absorptions at 3450 and 1680 cm^{-1} due to the alcohol and the amide carbonyl group, respectively.

4'-(*N*'-Acetylamino)benzyl *N*-(4-nitrophenyl) carbamate **(26)** was obtained in 69% yield after silica column purification and gave m.p. 200-201°C. The MS(FAB^-) spectrum showed m/z=328 corresponding to $M-H^+$ and the ^1H-NMR spectrum [EtOD/$(CD_3)_2SO$] showed a singlet (3H) at δ=2.09 ppm for the N-acetyl group, a singlet (2H) at 5.16 ppm due to the CH_2 group, two sets of AB quartets (4H each) centred at 7.78 and 7.93 ppm due to the aromatic protons, and a broad exchangeable singlet (2H) at 4.40 ppm due to the NH protons. The IR spectrum showed strong absorptions at 1722 and 1700 cm^{-1} due to the carbonyl groups of the carbamate and amide functions, respectively.

3.2.4.2 Decomposition

The *N*'-acetyl carbamate **(26)** is insoluble in water and either insoluble or poorly soluble in most organic solvents. DMSO is exceptional so most of the decomposition studies were carried out in aqueous DMSO (7:3, v/v) and a few in either DCM or THF.

Two sets of experiments were carried out. The first using HPLC detection to identify products, the second using UV-visible spectrophotometry for time dependent measurements.

3.2.4.2.1 HPLC studies

N'-Acetyl carbamate **(26)** (ca. $4x10^{-5}$M) was decomposed at 37°C over 24h in aqueous solutions of DMSO (7:3, v/v) at pH 2, 7 and 12, as well as in two organic solvents: DCM and THF. The products were assayed by HPLC against authentic 4-nitroaniline **(92)** (Rf=19.20 min), 4-aminobenzyl alcohol **(80)** (Rf=2.98 min), 4-(*N*-acetylamino)benzyl alcohol **(96)** (Rf=13.58 min) and the substrate itself **(26)** (Rf=24.01 min). Details of these experiments are given in Experimental (Section 5.4.6.2).

The HPLC detection was carried by UV at λ=249 nm. This wavelength was chosen because it corresponds to the λ_{max} for the decomposition products **(80)** and **(96)**. The HPLC assays for the various reactions are summarised in ***Table 3.1***. Both the products are identified and their relative concentrations reported.

In aqueous DMSO (7:3,v/v) at pH 2 and 7, **(26)** is relatively stable showing less than 10% decomposition at 37°C over 24h. The decomposition products identified by HPLC were 4-nitroaniline **(92)** (7-8%), 4-(*N*-acetylamino)benzyl alcohol **(96)** (*ca.* 1%) and 4-amino-benzylalcohol **(80)** (8-9%), all in small amounts. The solid residue formed was not identified.

In aqueous DMSO (7:3,v/v) at pH 12, decomposition is more extensive and the solution develops a deep yellow colour. After 24h at 37°C, only *ca.* 8% of the starting material remains, the major products being 4-nitroaniline **(92)** (90%) and 4-(*N*-acetyl-amino)benzyl alcohol **(96)** (58%), together with smaller amounts of 4-aminobenzyl alcohol **(80)** (*ca.* 11%). Formation of large amount of 4-nitroaniline explains the deep yellow coloration.

Spontaneous decomposition of **(26)** in THF and DCM is more extensive than in aqueous DMSO and after 24h at 37°C, the relative peak area of **(26)** was only 8% in DCM and 6% in THF. The same decomposition products were identified [**(80)**, **(96)** and **(92)**] but in different relative amounts.

The effect of solvent on the stability of **(26)** may relate to solubility differences.

Table 3.1

Stability of the N'-acetyl carbamate (26) at 37°C over 24h

Conditions	Condition of final reaction solution	Structure, Rf (min) and (% product relative to $[26]_{initial}$)
H_2O/DMSO (7:3,v/v) pH 2	Solid residue	**(26)** 24.01 (68)
		(80) 2.95 (9)
		(92) 19.20 (8)
		(96) 13.58 (1)
H_2O/DMSO (7:3,v/v) pH 7	Solid residue	**(26)** 24.01 (67)
		(80) 2.95 (8)
		(92) 19.20 (7)
		(96) 13.58 (1)
H_2O/DMSO (7:3,v/v) pH 12	Deep yellow solution, no solid residue	**(26)** 24.01 (8)
		(80) 2.95 (11)
		(92) 19.20 (90)
		(96) 13.58 (58)
DCM	Yellow solution, no solid residue	**(26)** 24.01 (8)
		(80) 2.95 (19)
		(92) 19.20 (86)
		(96) 13.58 (49)
THF	Yellow solution, no solid residue	**(26)** 24.01 (6)
		(80) 2.95 (39)
		(92) 19.20 (87)
		(96) 13.58 (31)

3.2.4.2.2 UV/visible spectrophotometric studies

The decomposition of carbamate **(26)** in aqueous DMSO (7:3, v/v) at pH 2, 7 and 12 and temperatures from 25°C to 70°C was also followed by UV/visible spectrophotometry of the reaction solutions between λ=240 and 500 nm at timed intervals. Usually, a stock solution

(10 μl) of **(26)** in DMSO (1.2 $x10^{-2}$M), was injected into a cuvette containing aqueous DMSO (7:3,v/v) ($3cm^3$) at pH 2, 7 or 12, adjusted with either NaOH (1M) or HCl (1M). The cuvette was placed in the temperature regulated compartment of the spectrophotometer, and the UV/visible spectrum recorded when required.

3.2.4.2.2.1 Reactions at 25°C

A plot of absorbance of the reaction solution against time is represented in *Fig. 3.1* for the three different conditions studied: a) neutral conditions; b) acid conditions; c) basic conditions, all at 25°C for 15h.

Both in neutral (pH 7) and in acidic (pH 2) conditions, the initial maximum absorbance is centred at λ=325 nm [*Fig 3.1* a) (pH 7) and b) (pH 2)]. As the reaction proceeds, the absorbance slightly decreases, but there is no shift in the position of λ_{max}. The decrease in the intensity of λ_{max} occurs with simultaneous formation of a white precipitate (this precipitate was not analysed).

In basic (pH 12) conditions, a different behaviour occurs. The initial λ_{max} at 395 nm shifts to 360 nm as the reaction proceeds (*Fig 3.1* c). The initial absorbance in basic conditions is similar to that observed initially in neutral and acidic conditions, but it does not decrease with time. Other major differences are the development of a deep yellow colour in the basic solution, in contrast to the colourless neutral and acid reaction solutions, and no evidence of a white precipitate.

3.2.4.2.2.2 Reactions at 70°C

The above procedure was also carried out keeping the reaction solutions at 25°C for 1h and then at 70°C for 48h.

As for 25°C, a similar behaviour was apparent for neutral and acidic conditions and a different behaviour in base. However, at 70°C decomposition is faster and more extensive.
For acidic and neutral conditions during the first hour at 25°C, the intensity of the initial λ_{max}=325 nm decreased, then increased as the temperature rose from 25 to 70°C, probably due to an increased solubility of **(26)**. Further, in neutral conditions after 49h, the λ_{max} shifts from 325 to 330 nm with a shoulder at λ_{max} > 360 nm. In acidic conditions after 49h, the λ_{max} shifts from 325 to 368 nm.

In basic conditions, the reaction proceeds faster than in neutral or acidic conditions. During the first 20 min at 25°C, the λ_{max} shifts from 395 to 360 nm, and after 48h at 70°C shifts from 360 to 384 nm.

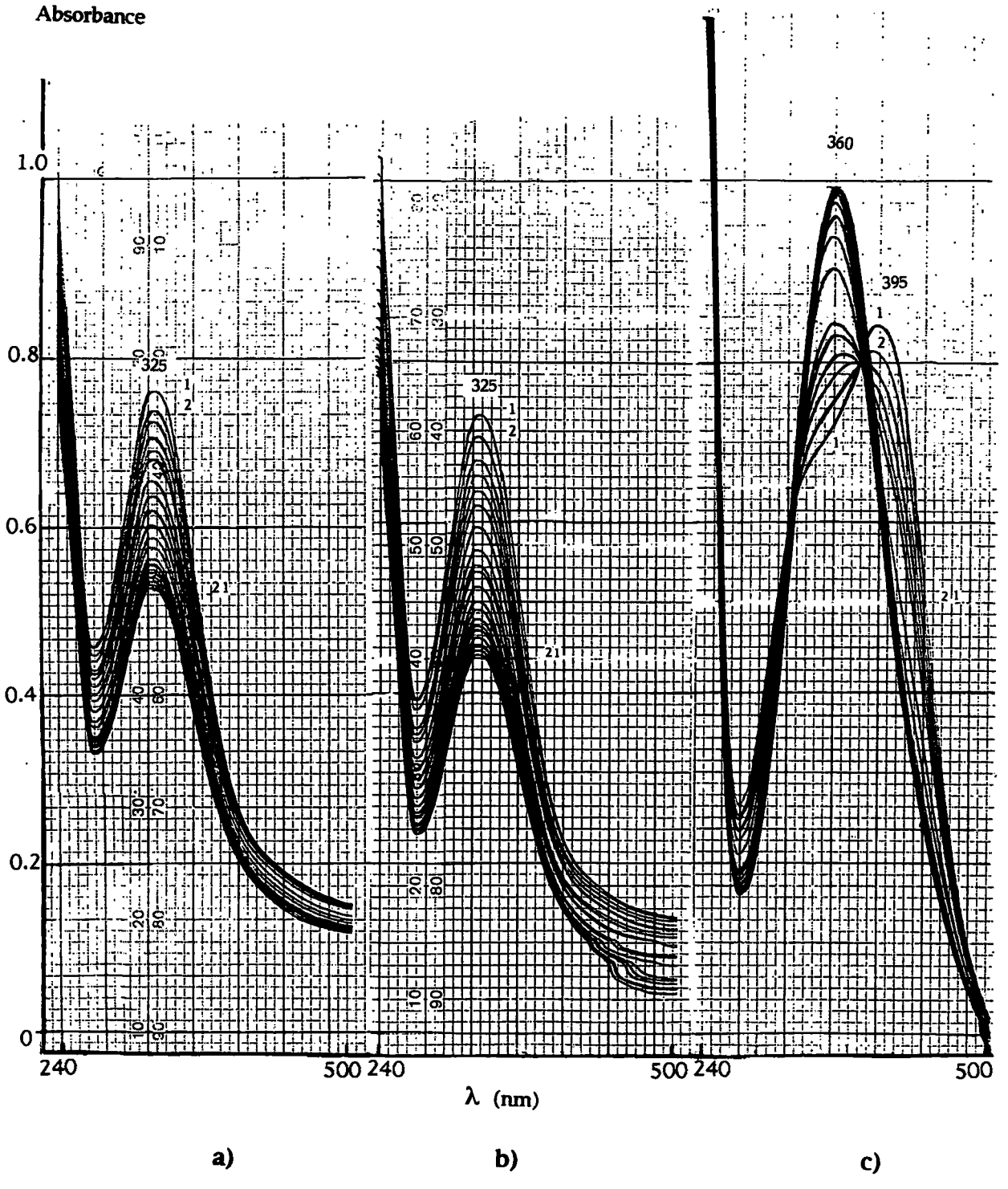


Figure 3.1 *UV/visible spectra for hydrolysis of (26) in aqueous DMSO (7:3,v/v) at 25°C for 15h: a) pH 7 b) pH 2 and c) pH 12 ; $[79]_{init.}$ ca. $4x10^{-5}$M; scans 1-6 (15 min interval), scans 7-21 (60 min interval)*

The UV spectra of 4-nitroaniline in aqueous DMSO (7:3,v/v) at 25°C and pH 2, 7 and 12 all show an absorbance maximum at λ=381 nm; the UV spectra of 4-nitroisocyanate under similar conditions show λ_{max} =342 nm at pH 2 and 7, and λ_{max}=348 nm at pH 12.

The UV-visible results suggest that even at 70°C over 48h, 4-nitroaniline is not formed in observable amounts in either neutral or acidic conditions. In basic conditions after heating the solution at 70°C over 48h, the final λ_{max}=384 nm suggests complete decomposition of carbamate **(26)** into 4-nitroaniline; however, at 25°C over 24h, this decomposition is not observable.

These results suggest that carbamate **(26)** is relatively stable in aqueous solutions at pH 7, pH 2 and 25°C.

3.2.4.2.2.3 Effect of adding excess acid to the basic solution and excess base to the acidic solution

The effect of pH change for the reaction solutions was also examined. The procedure was the same as that described previously (Section 3.2.4.2.2.1) and after adding substrate, the solutions were kept at 25°C for no longer than 10 min (*i.e.* before significant decomposition had occurred) before adding acid or base.

On adding excess HCl to the basic solution, the λ_{max} immediately shifted from 395 to 325 nm and the colour of the solution changed from deep yellow to colourless.

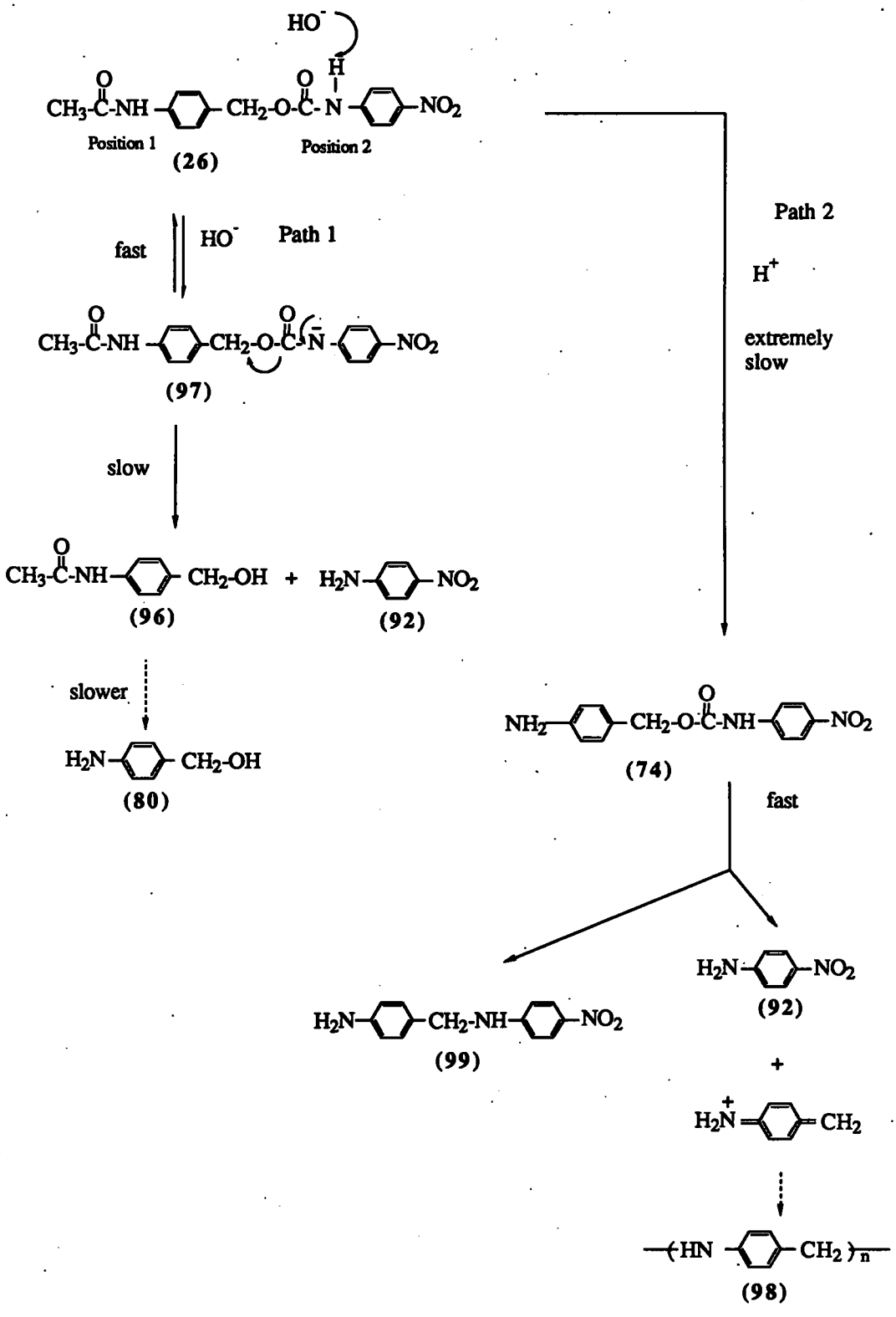
On adding excess of NaOH to the acidic solution, an immediate shift of λ_{max} from 325 to 400 nm occurred with simultaneous development of a deep yellow colour.

These results suggest an acid-base equilibrium at pH 12, involving **(26)** and **(97)** (*Scheme 3.12*).

3.2.4.2.3 Conclusions

Both the HPLC identification of the final reaction products and the UV-visible studies (pointing to a possible acid-base equilibrium at pH 12) suggest that hydrolysis of **(26)** is fast and base-catalysed, leading to 4-(*N*-acetylamino)benzylalcohol **(96)** and 4-nitroaniline **(92)** as major products (*Path 1* in *Scheme 3.12*). 4-(*N*-Acetylamino)benzylalcohol **(96)** hydrolyses further to 4-aminobenzylalcohol **(80)**.

In neutral and acidic conditions, decomposition is very slow and the main hydrolysis products are 4-aminobenzylalcohol **(80)** and 4-nitroaniline **(92)**. A possible explanation for their formation may be hydrolysis of **(26)** to **(96)** and a further hydrolysis of **(96)** to **(80)**. The initial hydrolysis of the amide function in **(26)** leads to compound **(74)** which gives a polymer of general structure -(-NH-C_6H_4-CH_2-)$_n$- **(98)** and compound **(99)** rather than 4-amino-benzylalcohol **(80)** (*Path 2* in *Scheme 3.12*) as discussed in Section 3.3.4.



Scheme 3.12 Hydrolysis of (26) in aqueous DMSO (7:3, v/v) at pH 2, 7 or 12 and in DCM or THF at 37°C

3.3 The 4'-amino carbamate (74)

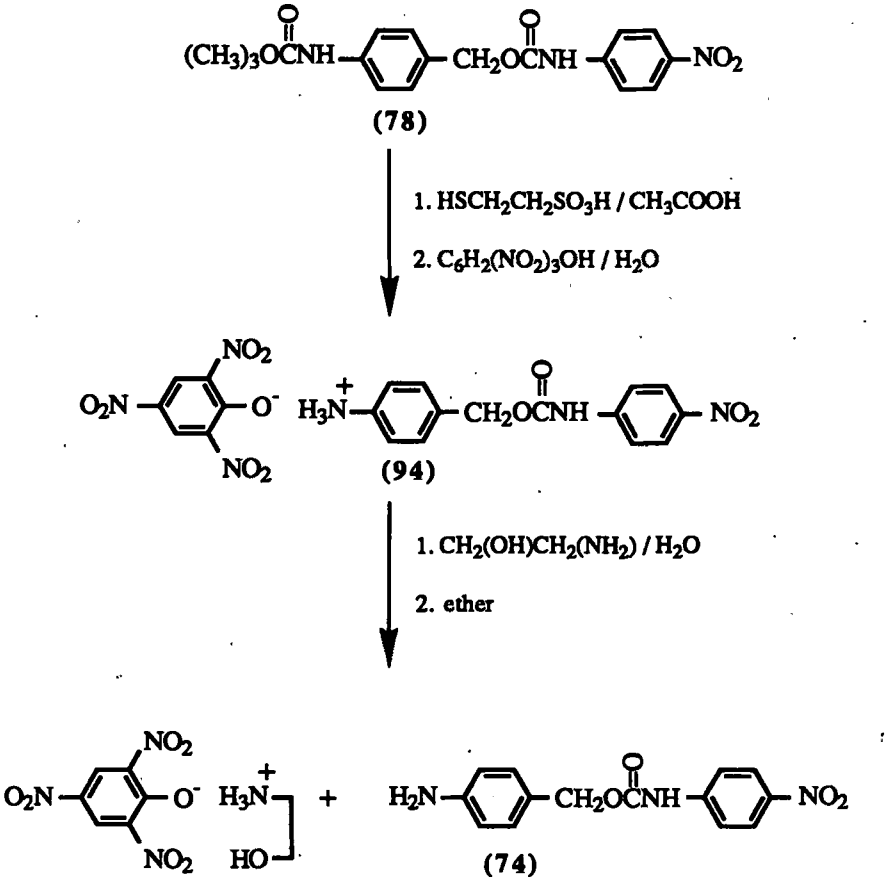
3.3.1 Synthesis

The 4'-aminobenzyl *N*-(4-nitrophenyl) carbamate **(74)** was obtained via the N'-BOC derivative **(78)** as previously outlined by the following sequence of steps: (1) Mild hydrolysis of **(78)** in THF using 20% (w/v) 2-mercaptoethanesulphonic acid in glacial acetic acid at 25°C, followed by immediate reaction with aqueous picric acid to form the picrate salt **(94)**, in 84% yield. (2) Conversion of the isolated picric salt to the neutral carbamate by treatment with 10% (w/v) aqueous ethanolamine, which removes the picrate moiety by formation of the more stable picric salt of ethanolamine **(100)**. Compound **(74)** was then extracted into ether. This sequence is described by *Scheme 3.13*.

Silica tlc of the ether extract containing **(74)** showed that the product was impure and that the impurities increased quickly on standing. This suggests that **(74)** is very labile. It was therefore purified by silica column chromatography using ether immediately after extraction. Three separate columns, however, were required to obtain a reasonably pure sample of compound **(74)**. The major impurities showed Rf=0.2 (4-nitroaniline **(92)**) and Rf=0.35 (unidentified); the required compound **(74)** gave Rf=0.15. After isolation, compound **(74)** was identified by MS and NMR, as described below.

4'-Aminobenzyl *N*-(4-nitrophenyl) carbamate, picrate salt **(94)** was obtained in 84% yield and gave m.p. 120-122°C. The MS(FAB$^+$) spectrum shows m/z=289 corresponding to $NO_2C_6H_4NHC(O)OCH_2C_6H_4NH_3^+$ and MS(FAB$^-$) spectrum shows m/z=228 corresponding to the picrate anion $(O_2N)_3C_6H_2O^-$. The ^{1}H-NMR ($(CD_3)_2SO$) shows a singlet (2H) at δ=5.26 ppm consistent with the benzylic CH_2 group, two sets of AB quartets (4H each and both with J=2 Hz) at 6.62-7.45 ppm and 7.71-8.22 ppm, corresponding to the 4-nitroaryl and 4-phenylene moieties, respectively, and a singlet (2H) at 8.63 ppm corresponding to the aromatic protons of the picrate anion; the broad, exchangeable singlet at 10.08 ppm and multiplet at 7.43 ppm are attributed to the NH protons. The IR spectrum shows a strong absorption at 1760 cm^{-1} due to the carbamate carbonyl group.

4'-Aminobenzyl *N*-(4-nitrophenyl) carbamate **(74)** was obtained reasonably pure in 55% yield and gave m.p. 110-140°C (decomp). The MS(FAB$^-$) spectrum shows m/z=286 due to M-H$^+$ and the ^{1}H-NMR spectrum [$CDCl_3$+ $(CD_3)_2SO$] shows a singlet (2H) at δ=5.04 ppm corresponding to the benzylic CH_2 group, two sets of AB quartets (4H each) at 6.65-7.17 ppm



Scheme 3.13 Synthesis of 4'-amino carbamate (74)

with J=2.1 Hz, and 7.70-8.11 ppm with J=2.3 Hz, as expected for the aromatic protons; 3 broad, exchangeable singlets at 3.26, 4.47 and 10.10 ppm are attributed to NH protons. The IR spectrum shows absorptions at 1760 cm^{-1} and 3400 cm^{-1}, of strong and medium intensity, due to the carbamate carbonyl and the NH_2 groups, respectively.

3.3.2 Spontaneous decomposition of (74)

From the difficulties encountered in purification, 4'-aminobenzyl *N*-(4-nitrophenyl) carbamate (74) appeared to be a labile compound. It was therefore decided to study its spontaneous decomposition first. Accordingly, compound (74) dissolved in DMSO/chloroform (3:7, v/v) was kept at 65°C, and the reaction mixture monitored by silica tlc, GC, GC-MS and ^{1}H-NMR over 48h.

Silica tlc, using ether as eluent, of the reaction mixture showed complete disappearance of (74) after 2h at 65°C, to form three major products with Rf=0.7 (same Rf as 4-nitroaniline (92)), Rf=0.5 and Rf=0.3. Silica tlc of the reaction mixture at later times (5h, 10h, 48h) did not show any major changes.

Capillary GC analysis of the reaction mixture was also carried out using on-column injection (to minimise decomposition) on BP5 and BP20 columns, the former with a N-specific detector. Under the temperature programme employed, authentic compounds showed retention times listed on *Table* 3.2 (carbamate (74) is not eluted on either BP5 or BP20 columns).

After 2h at 65°C, the reaction mixture showed two peaks only on the BP5 column, at Rf=11.91 min (4-nitroaniline) and at Rf=24.50 min (unidentified). After 10h and 48h at 65°C, the reaction mixture gave similar chromatograms on the BP5 column with a small increase in the peak at Rf=24.50 min.

Similarly, assays with the BP20 column showed one signal with Rf=13.0 min (4-nitroaniline) after 2h at 65°C, with no further changes for longer reaction times.

Significantly, the GC assays gave no evidence for the formation of 4-aminobenzyl alcohol (80) or 4-toluidine (89) products.

Table 3.2

Capillary GC retention times (Rf) for authentic compounds (80), (89) and (92)

Compound		Rf / min	
	Column:	BP5	BP20
4-Aminobenzyl alcohol	(80)	8.26	Not eluted
4-Toluidine	(89)	3.85	4.12
4-Nitroaniline	(92)	11.91	13.0

Samples of the reaction mixture after 2h and 48h at 65°C were also analysed by GC-MS using a BP5 column, on-column injection, and a similar temperature programme to the one for the regular GC assays. These gave 2 major signals at Rf=13.8 min (larger), and Rf=25.6 min, which correspond to the two peaks in the regular GC traces at Rf=11.91 min and Rf=24.50 min. The first showed m/z=138 corresponding to M^+ for 4-nitroaniline (92) and the second showed m/z=243 corresponding to M^+ for *N*-(4-aminobenzyl)-4-nitroaniline (99).

Significantly, no evidence was found for the formation of either 4-aminobenzylalcohol **(80)** or 4-toluidine **(89)**.

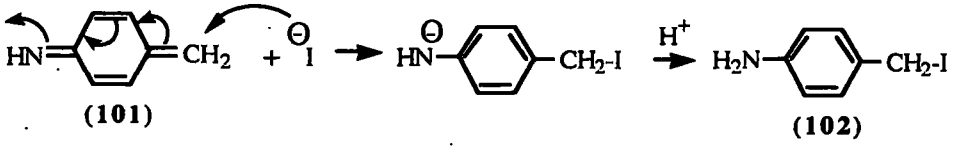
An MS(EI) assay of the solid residue formed by the reaction in DMSO/$CHCl_3$ (7:3, v/v) at 65°C after 5h showed the presence of several strong peaks with m/z at multiple values of 105. This is consistent with the formation of a polymeric product, with a $-(-NH-C_6H_4-CH_2-)_n-$ structure, by self condensation of the methide fragment from the substrate **(74)**.

In a separate experiment, carbamate **(74)** in $(CD_3)_2SO$/$CDCl_3$ (3:7,v/v) was heated at 65°C in a sealed NMR tube. The ^{1}H-NMR spectra recorded after 2h showed that the signals for **(74)** had entirely disappeared (***Figure 3.2***). New signals then apparent were a broad singlet (2H) at δ=4.41 ppm (probably due to a benzylic CH_2 group), a second broad singlet (2H) at 6.12 ppm, a third broad singlet (2H) at 6.61 ppm, an AB quartet (4H) at 6.63-7.95 ppm (J=2.0 Hz), and two broad singlets (1H each) at 6.94 and 7.07 ppm. Spectra of the reaction mixture recorded after 5h,10h, 24h and 48h at 65°C were similar to that after 2h, despite formation of a precipitate in the NMR tube after 5-6h. These ^{1}H-NMR spectra therefore confirm the chromatography experiments ie. compound **(74)** decomposes over 2h at 65°C to give products that are not transformed by further heating.

3.3.3 Spontaneous decomposition in the presence of LiI

In view of the above results and especially the formation of polymeric material, attempts were made to trap the 4-amino methide fragment **(101)** by carrying out the decomposition of **(74)** in DMSO/chloroform (3:7, v/v) at 65°C and in the presence of equimolar LiI.

The choice of LiI as a trapping agent relates to its thermal stability and the strong nucleophilicity of I^-. Formation of 4-α-iodomethylaniline **(102)** was anticipated (***Scheme 3.14***). This compound is not described in the literature, but the related compounds 4-α-cloromethylaniline,[183] 4-α-fluoromethylaniline [184] and 4-α-bromomethylaniline [185] have been reported.



(101) **(102)**

Scheme 3.14 *The intermediate species being trapped by the nucleophile I^-*

The reaction products were examined by silica tlc (using ether as eluent), GC/MS and ^{1}H-NMR in similar ways to those described in Section 3.3.2. Briefly, identical findings apply to those obtained without LiI, and no new products were formed:

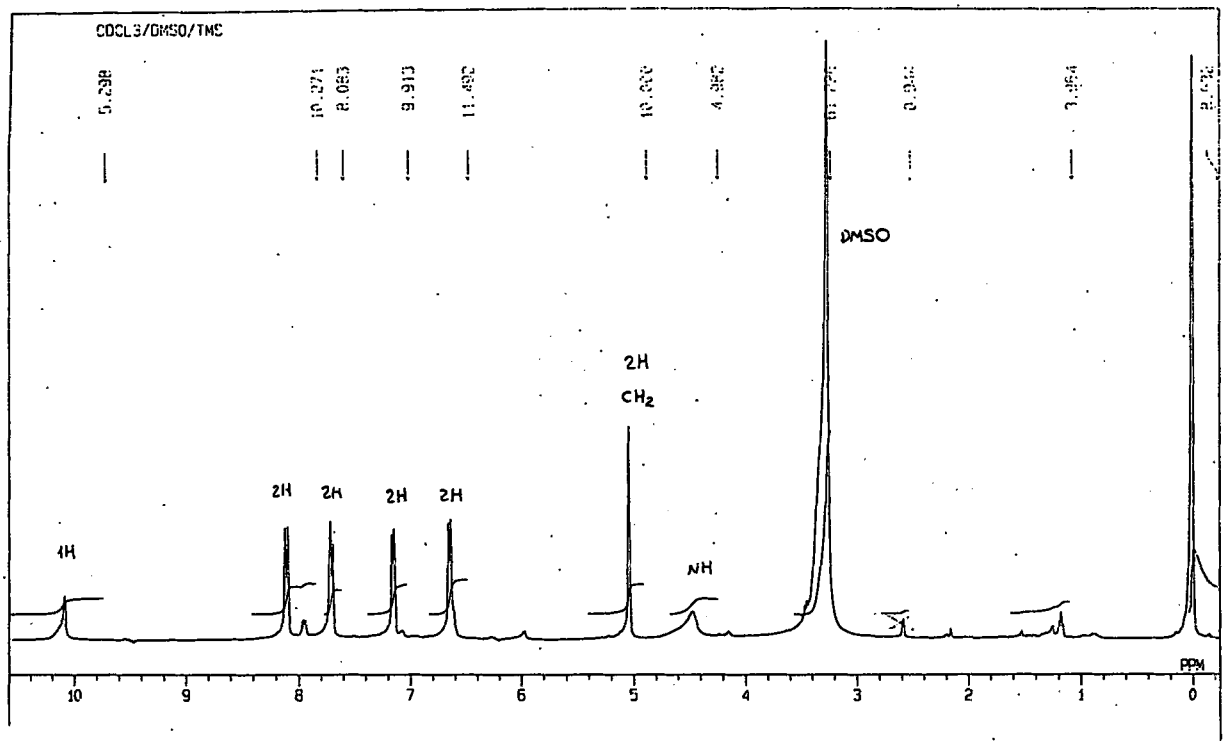
After 2h at 65°C, the substrate (74) completely disappeared. At this point, silica tlc of the reaction solution (ether eluent) gave 2 spots as before, with Rf=0.5 and 0.7, and the GC-MS was also identical with and without added LiI: the same two major peaks were observed at Rf=13.2 min and Rf=24.5 min showing m/z=138 and m/z=244, respectively.

After 3h at 65°C, the reaction was worked-up to isolate products by vacuum evaporating the solvent and extracting the solid residue with DCM. The DCM was then vacuum evaporated, the residue suspended in ethyl acetate and applied to a silica preparative plate. Three major fractions were obtained with Rf=0.6, 0.4 and 0.2. Each fraction was removed from the plate, extracted into DCM, the solvent vacuum evaporated and the residues analysed by ^{1}H-NMR and MS:

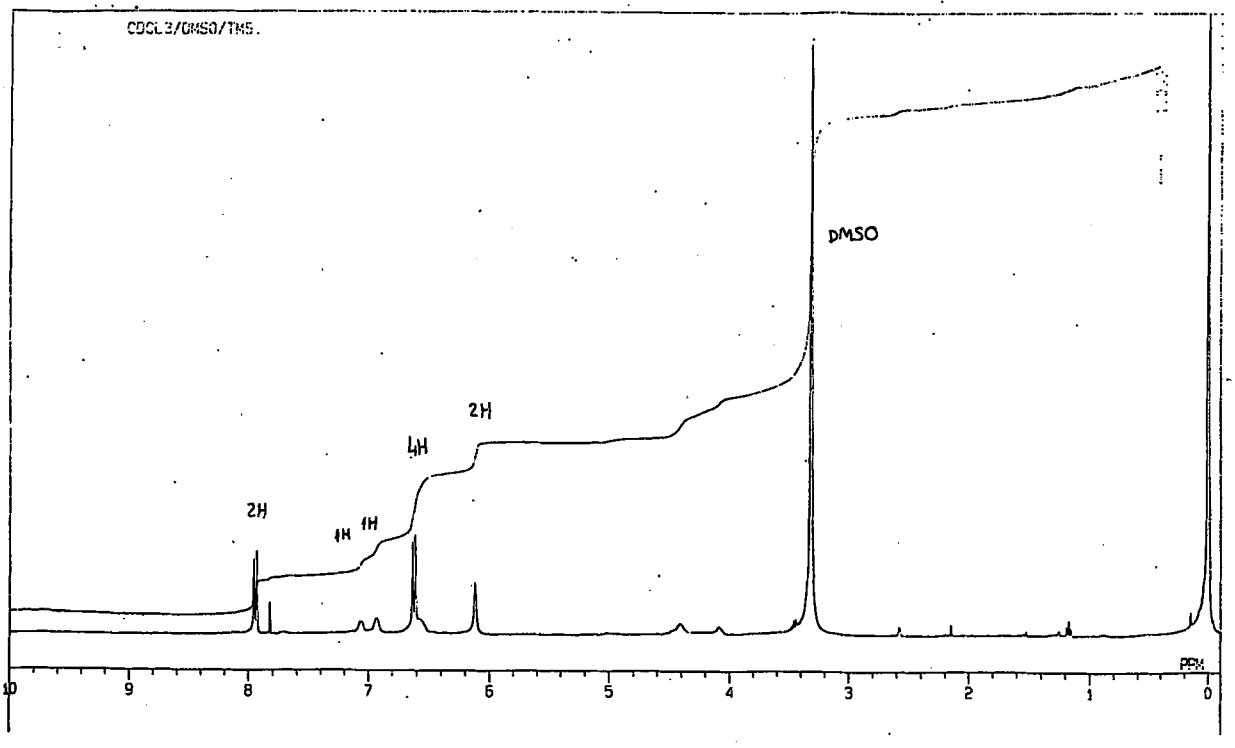
Fraction 1 with Rf=0.6 gave both MS(EI) spectrum (ie. m/z=138) and ^{1}H-NMR spectrum identical to that of 4-nitroaniline (92) (*Figure 3.3*). It also had a GC retention time identical with 4-nitroaniline.

Fraction 2 with Rf=0.4 gave both MS(FAB$^+$) spectrum (ie. m/z=244) and ^{1}H-NMR spectrum consistent with *N*-(4'-aminobenzyl)-4-nitroaniline (99) (*Figure 3.4*) (see Section 3.3.2).

Fraction 3 with Rf=0.2 gave MS(FAB$^+$) spectrum with strong peaks at m/z= multiple values of 105 and a ^{1}H-NMR spectrum in $CDCl_3/(CD_3)_2SO$, showing a doublet at δ=2.6 ppm and an AB quartet at 7.6-7.8 ppm (*Figure 3.5*). The ^{13}C-NMR spectrum shows only two signals at 78.36 (triplet) and 39.92 ppm (septet). The MS findings suggest a polymeric -(-NH-C_6H_4-CH_2-)$_n$- structure for this fraction.



a)



b)

Figure 3.2 *^{1}H-NMR of (74) : a) immediatly after dissolving in $CDCl_3/(CD_3)_2SO$ without heating; b) after heating at 65°C over 2h*

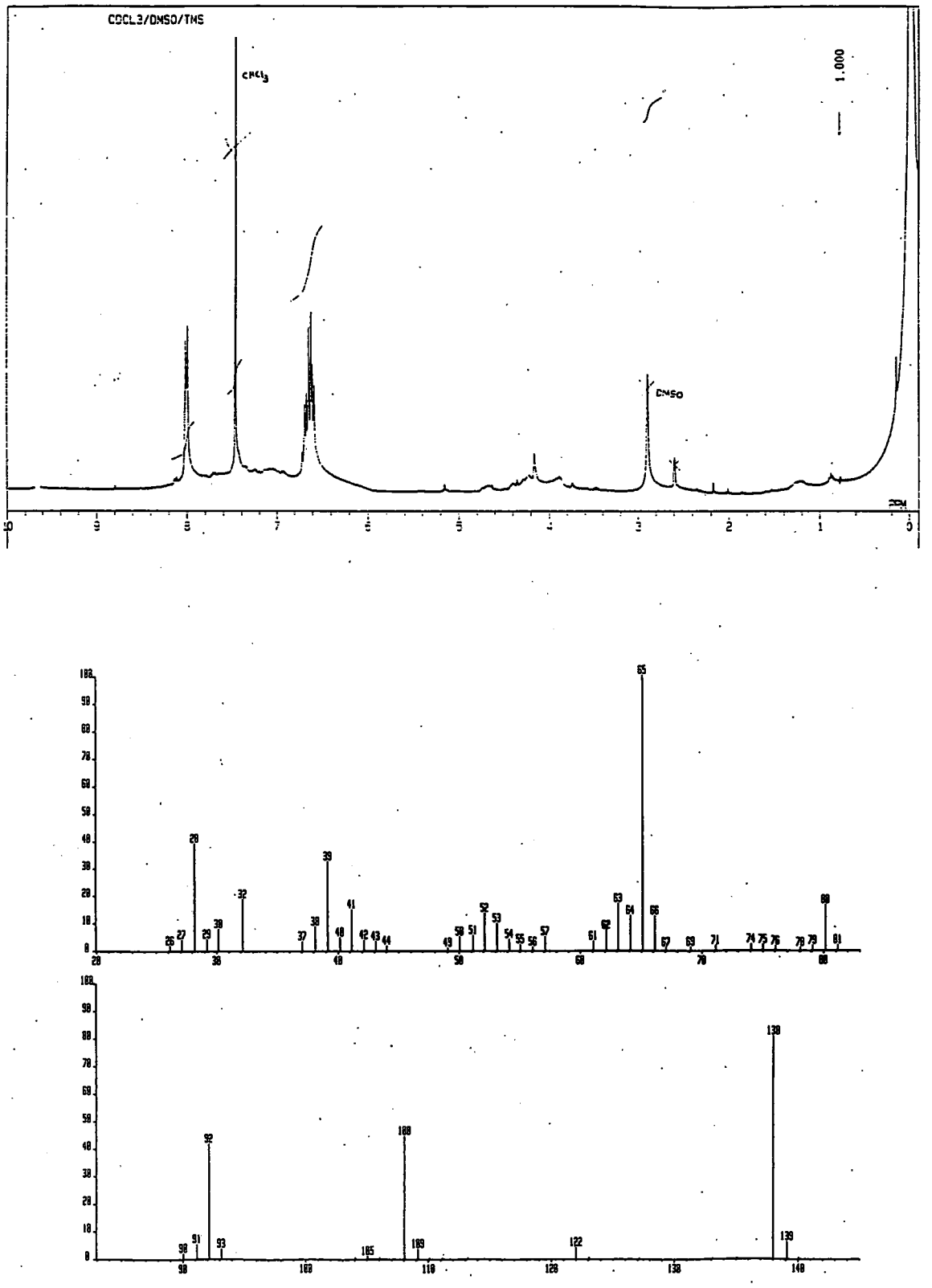


Figure 3.3 *^{1}H-NMR and MS(EI) of the decomposition products from (74) in DMSO/$CHCl_3$ (3:7, v/v) in the presence of equimolar LiI, at 65°C for 24h - Fraction 1*

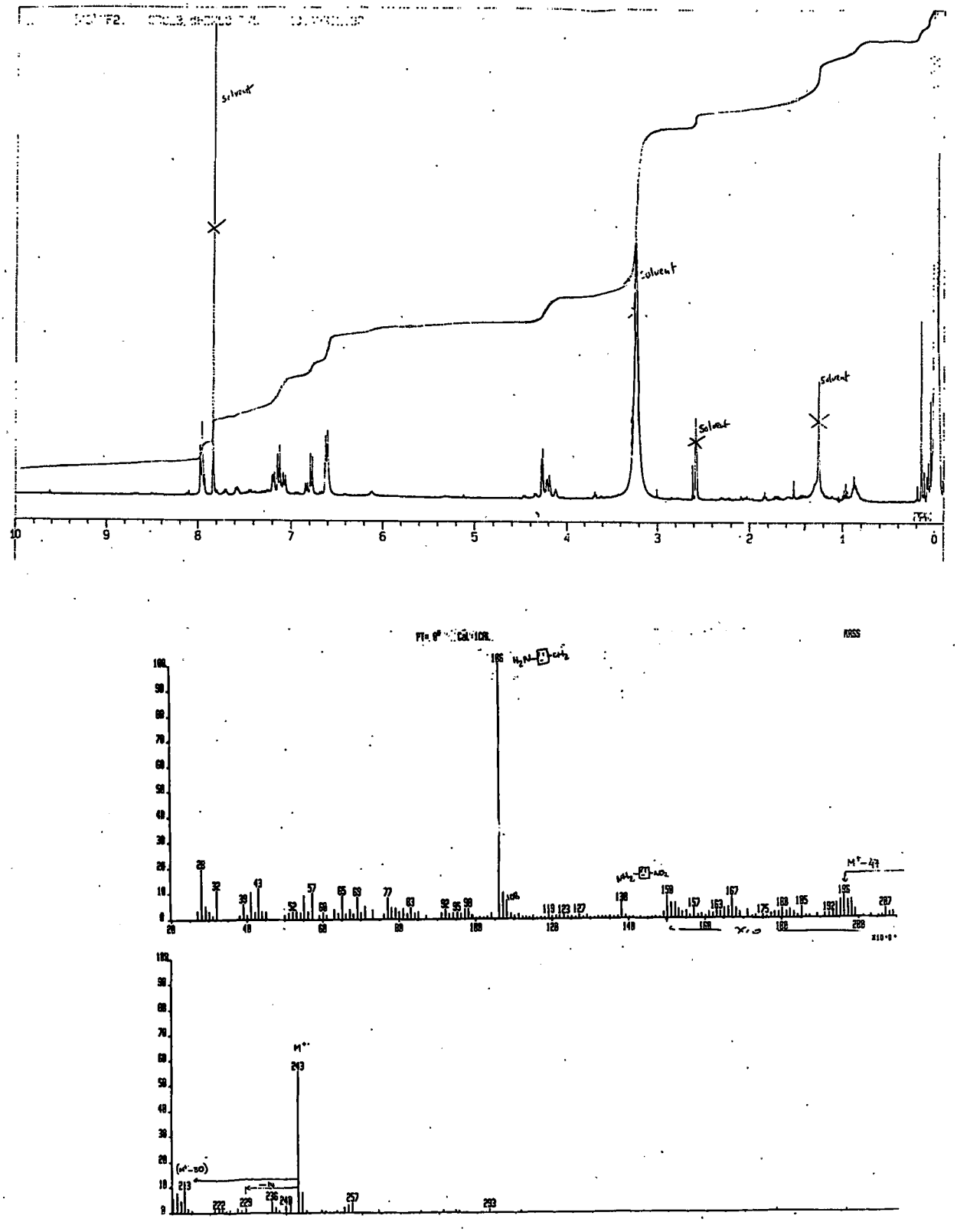


Figure 3.4 *^{1}H-NMR and MS(EI) of the decomposition products from (**74**) in DMSO/$CHCl_3$ (3:7, v/v) in the presence of equimolar LiI, at 65°C for 24h - Fraction 2*

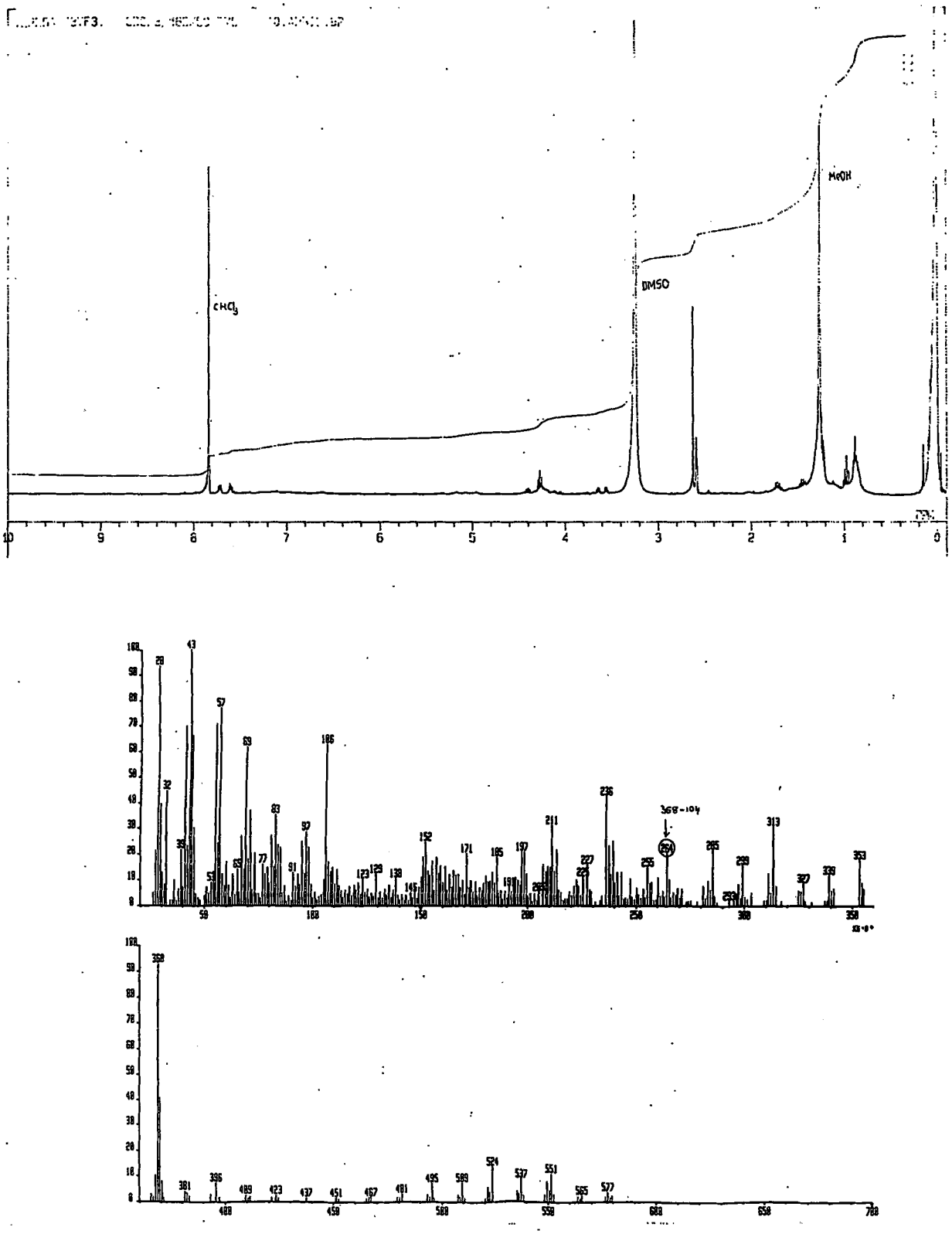
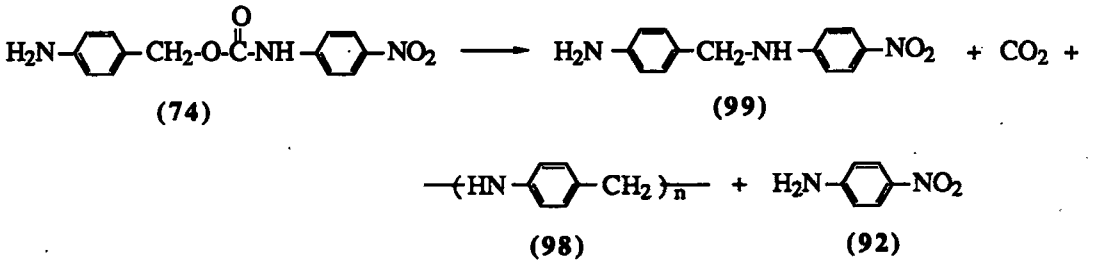


Figure 3.5 ^{1}*H-NMR and MS(EI) of the decomposition products from* (**74**) *in DMSO/CHCl*$_3$ *(3:7, v/v) in the presence of equimolar LiI, at 65°C for 24h - Fraction 3*

3.3.4 Discussion

The above results suggest that after 2h at 65°C, carbamate **(74)** completely transforms into 3 products, identified as *N*-(4'-aminobenzyl)-4-nitroaniline **(99)**, 4-nitroaniline **(92)**, and a polymer having the general structure -(-NH-C_6H_4-CH_2-)$_n$- **(98)** (*Scheme 3.15*).

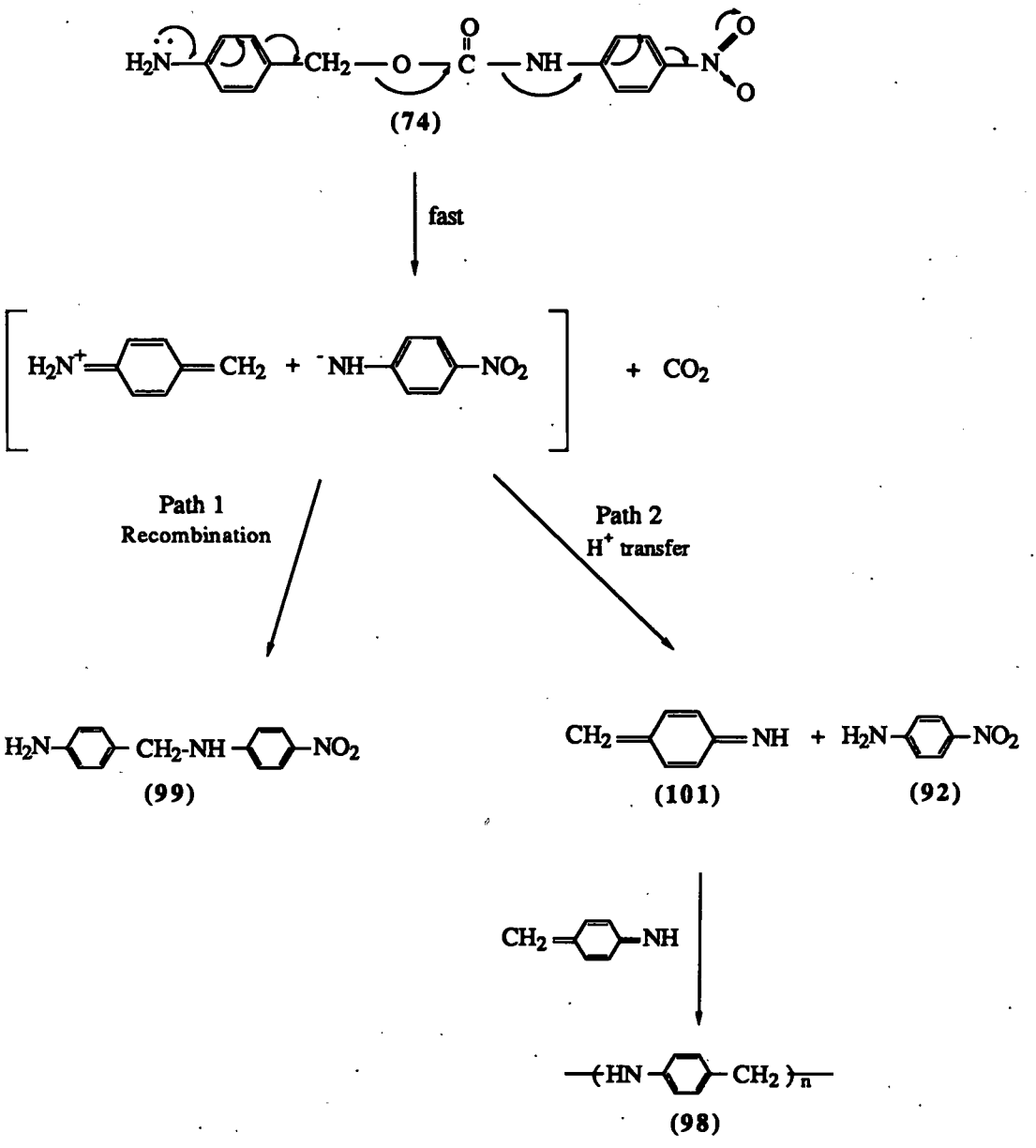


Scheme 3.15 Spontaneous decomposition of (74) in chloroform/DMSO (7:3,v/v) at 65°C

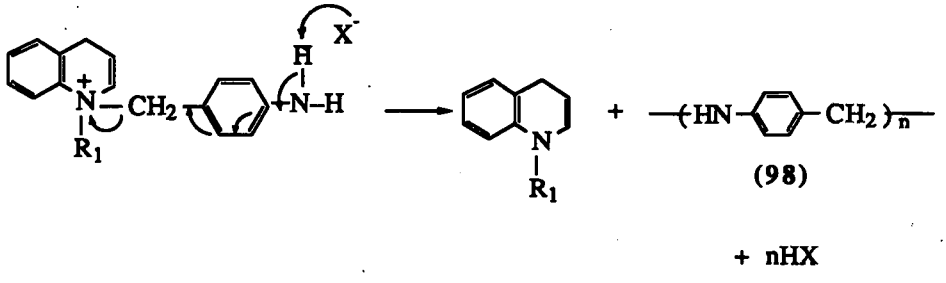
Insufficient information was obtained to deduce the mechanisms by which these products form. It is conceivable, however, that carbamate **(74)** thermally extrudes CO_2 in DMSO/$CHCl_3$ mixtures, to give a caged pair of ions which can either recombine (*Path 1* in *Scheme 3.16*) to give the *N*-(4'-aminobenzyl)-4-nitroaniline **(99)**, or by H^+-transfer (*Path 2* in *Scheme 3.16*) to generate 4-nitroaniline **(92)** and the aryliminomethide **(101)**. Subsequent reactions outside the solvent cage by **(101)** could generate the polymeric material **(98)** (*Scheme 3.16*).

The formation of 4-nitroaniline **(92)** could relate to simple hydrolysis of the carbamate **(74)**. However, the reaction medium contains no water and there is no evidence of 4-aminobenzyl alcohol **(80)**, the expected co-product of an hydrolysis reaction.

The formation of polymeric substances from self-condensation of an aryliminomethide intermediate has precedence in the decomposition of 4-aminobenzylquinolinium salts (*Scheme 3.17*).[86] The failure to trap the aryliminomethide by added I^-, however, is difficult to rationalise on the limited information available.



Scheme 3.16 Thermal decomposition of (74)



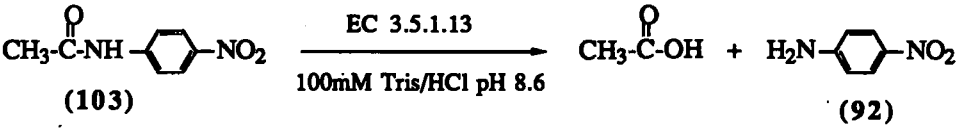
Scheme 3.17 The model reaction of aminobenzylquinolinium salts

3.4 Evaluation of *N'*-acetyl carbamate (26) as a substrate for acylarylamidase

Because the 4'-(*N'*-acetylamino)benzyl *N*-(4-nitrophenyl) carbamate **(26)** is reasonably stable in aqueous DMSO (7:3, v/v) (< 10% decomposition at pH 7 and 37°C over 24h), it was briefly investigated as a potential substrate for the enzyme acylarylamidase E.C. 3.5.1.13.

3.4.1 UV-visible procedures

Initially, the acylarylamidase enzyme was tested in tris/HCl buffers (100 mM, pH 8.6) at 37°C against 10^{-5}M 4-nitroacetanilide **(103)** as a model substrate. The reactions were carried out by UV-vis spectrophotometry (λ=250-450 nm), monitoring the release of 4-nitroaniline **(92)** (*Scheme 3.18*) from solutions in the thermostatted cuvette at 37°C, both in the presence and absence of acylarylamidase (2.7×10^{-4} units), as described in the Experimental.



Scheme 3.18 Model reaction of (103) as an acylarylamidase substrate

The variation of the UV-visible absorbances with time are shown as *Figure 3.6*, where the intervals between successive scans is 4 min (scan 12 after 1.5h).

The spectra in *Figure 3.6* a shows that 4-nitroacetanilide (λ_{max}=315 nm) hydrolyses rapidly in the presence of acylarylamidase E.C. 3.5.1.13 to release 4-nitroaniline (λ_{max}= 380 nm). A well-defined isosbestic point at λ=342 nm is apparent, indicating a clean conversion of 4-nitroacetanilide into 4-nitroaniline.

The spectra in *Figure 3.6* b show that release of 4-nitroaniline in the absence of the enzyme, is *ca.* 2×10^3 fold slower. An isosbestic point at λ=340 nm is also apparent.

The results confirm the procedure for testing substrate and that the sample of acylarylamidase is active.

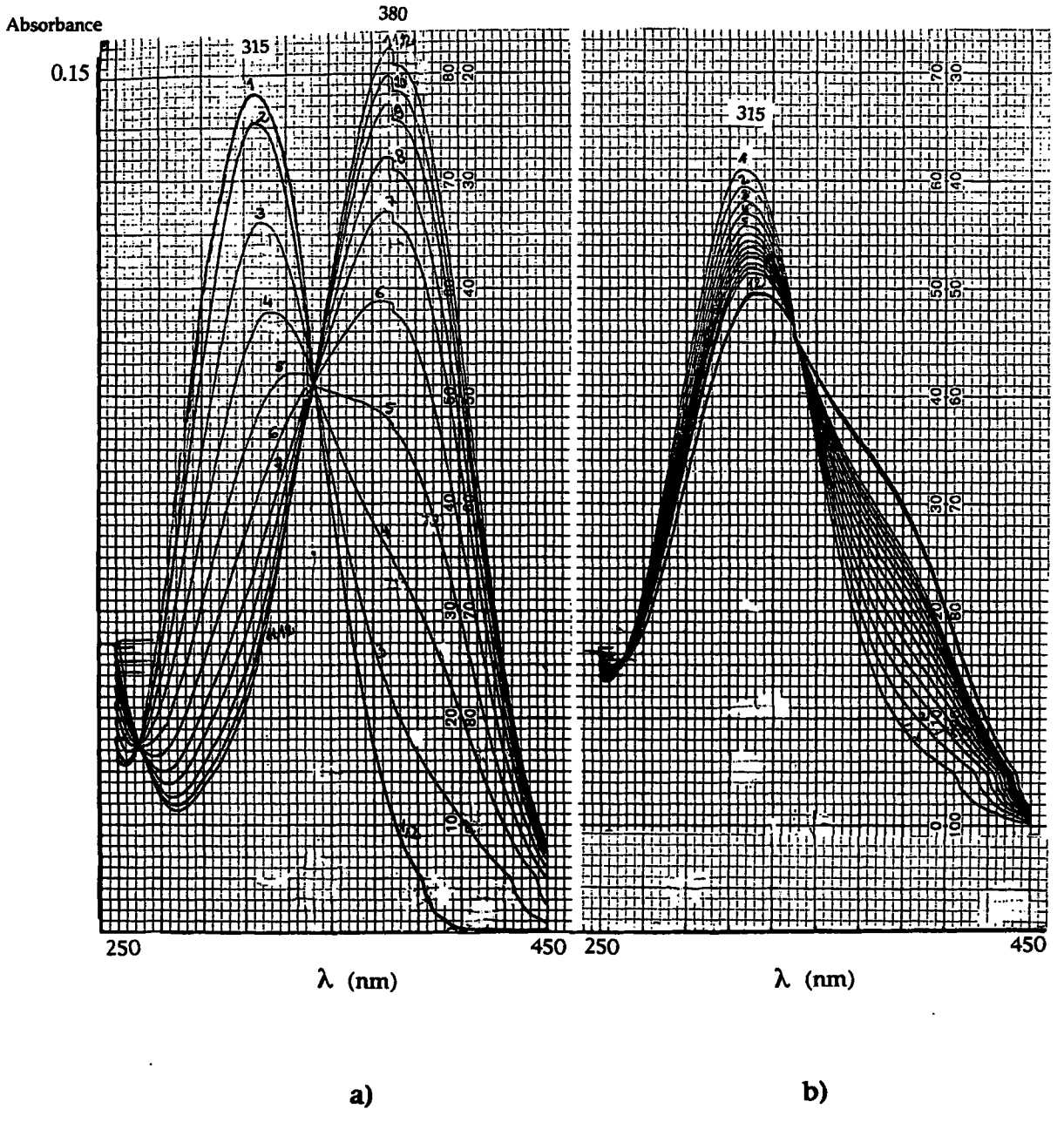


Figure 3.6 Decomposition of **(103)** *[1x10^{-5}M] in tris/HCl buffer at pH 8.6 and 37°C over 1.5 h: a) with acylarylamidase (18x10^{-5}units/cm^3); b) without enzyme scans 1-11 (4 min interval), scan 12 after 1.5h*

Carbamate **(26)** was tested as a substrate for acylarylamidase E.C. 3.5.1.13 in the same way as 4-nitroacetanilide. Thus, **(26)** (10^{-5}M) was reacted with acylarylamidase (2.7 x 10^{-4} units) in tris/HCl buffers at pH 8.6 and 37°C in the thermostatted cuvette of the spectrophotometer, and the spectrum scanned at intervals of 4 min (scan 11 after 1.5h). The

spectra, both with and without acylarylamidase, are shown in *Figure 3.7.* It is clear that carbamate **(26)** decomposes much faster in the presence of acylarylamidase, but the UV spectra over 1.5h show only a poorly defined isosbestic point (*Figure 3.7* **a**). This suggests that decomposition of **(26)** proceeds by more than one pathway. There is a slow change in the spectra of **(26)** in the absence of acylarylamidase (*Figure 3.7* **b**), which mainly reflects slow precipitation of the substrate from the reaction solution.

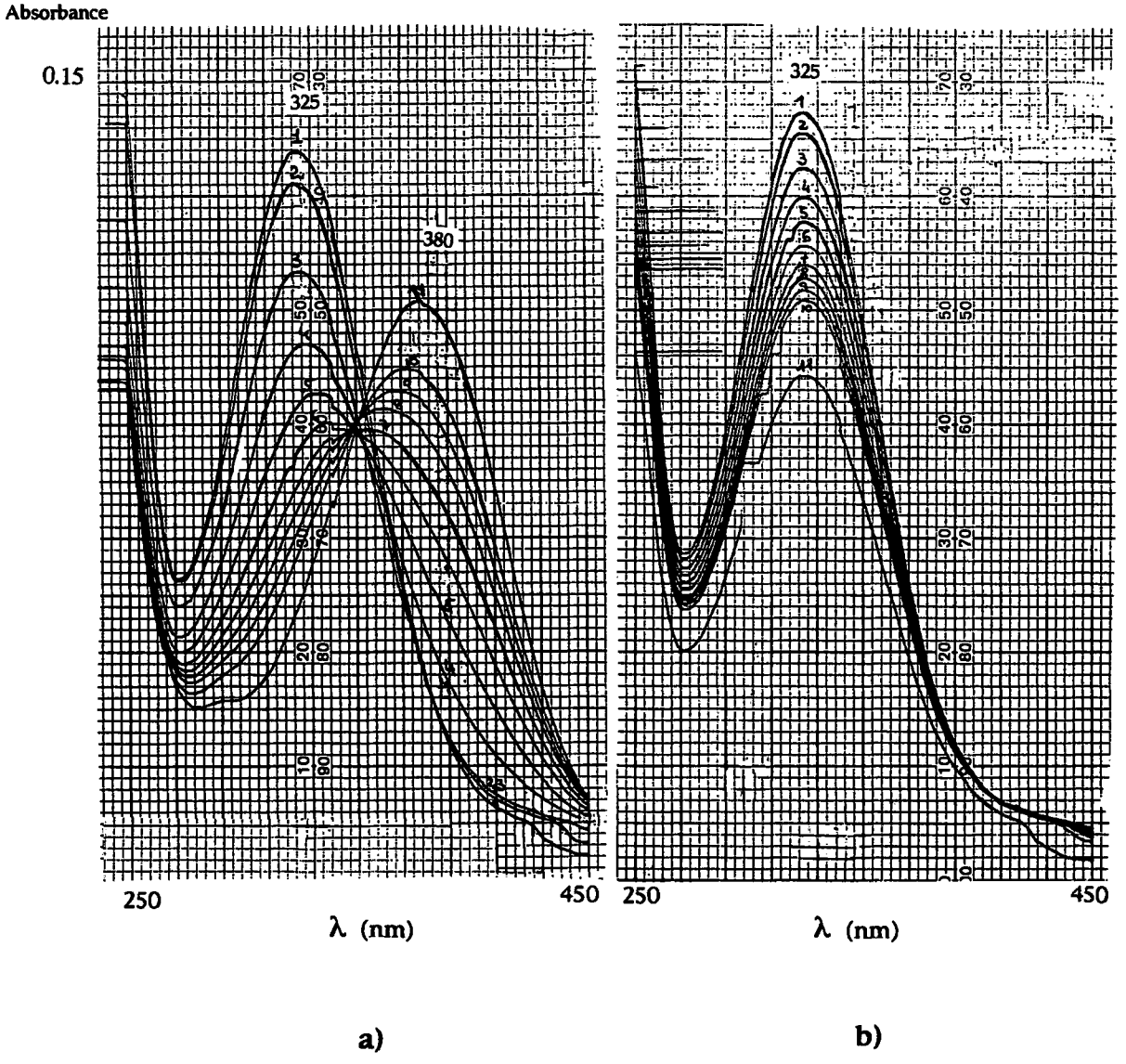


Figure 3.7 Decomposition of (26) [1x10^{-5}M] in tris/HCl buffer at pH 8.6 and 37°C over 1.5h : a) with acylarylamidase (18x10^{-5} units/cm^3), b) without enzyme; scans 1-10 (4 min interval), scan 11 after 1.5h

Approximate half-lives from the spectrophotometric data for the hydrolysis of 4-nitroacetanilide **(103)** and the *N'*-acetylcarbamate **(26)** in the presence and absence of acylarylamidase enzyme E.C. 3.5.1.13 are listed in ***Table 3.2.*** Insufficient time was available to carry out further quantitative experiments to determine V_{max} and K_m values for the substrate-enzyme combinations. Nonetheless, the data in ***Table 3.3*** show that N-arylcarbamate **(26)** is a substrate for the acylarylamidase E.C. 3.5.1.13, almost as good as 4-nitroacetanilide **(103)**.

Table 3.3
Approximate half-lives for the decomposition of (26) and (103) [$1x10^{-5}M$] in tris/HCl buffers at pH 8.6 and 37°C

Compound		Enzyme	Half-life
Carbamate	**(26)**	none	> 450 h
Carbamate	**(26)**	$18x10^{-5}$units/cm^3	12 min
4-Nitroacetanilide	**(103)**	none	> 400 h
4-Nitroacetanilide	**(103)**	$18x10^{-5}$units/cm^3	11 min

3.4.2 HPLC procedures

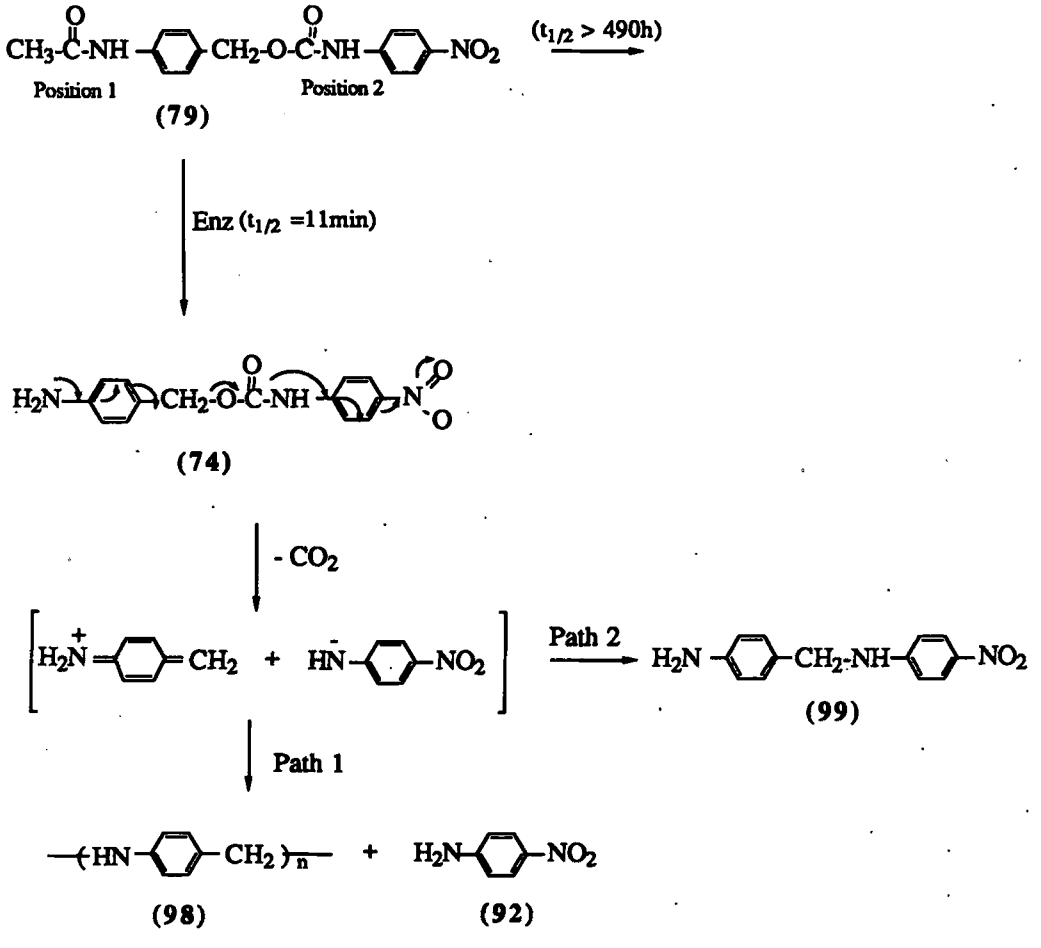
The decomposition of carbamate **(26)** by acylarylamidase E.C. 3.5.1.13 in tris/HCl buffer at pH 8.6 and 37°C was also followed by analytical HPLC. Products were identified by retention time against authentic 4-nitroaniline **(92)** (Rf=19.20 min), 4-aminobenzyl alcohol **(80)** (Rf=2.98 min) and 4-acetamidebenzylalcohol **(96)** (Rf=13.58 min). Reactions were carried out in a volumetric flask immersed in a thermostatted bath at 37°C, and aliquots of the reaction solution were withdrawn and injected at timed intervals (see Experimental).

The HPLC assay of the reaction solution just before the addition of the enzyme, showed only one peak (Rf= 24.01min) corresponding to *N'*-acylcarbamate **(26)**. One minute after addition of the enzyme, the HPLC assay showed a peak of reduced area at Rf=24.01min, plus a new peak at Rf= 19.20min corresponding to 4-nitroaniline. After 3 min, a second new peak at Rf=11.9 min (not identified) appeared in the HPLC chromatogram, along with the peaks due to 4-nitroaniline and **(26)**. An HPLC assay after 15 min, showed complete disappearance of the starting material **(26)**, a large peak at Rf=19.20 min due to 4-nitroaniline and a small peak at Rf=11.90 min [not identified, but probably due to N-(4-aminobenzyl)-4-nitroaniline **(99)**].

Neither 4-aminobenzyl alcohol **(80)** nor 4-(N-acetylamino)benzyl alcohol **(96)** were detected as reaction products at any stage of the reaction.

3.4.3 Conclusions

Both the HPLC assays and the absence of a good isosbestic point in the UV-visible spectra of the reaction solutions suggest that decomposition of *N'*-acylcarbamate **(26)** may proceed via 2 concurrent pathways as represented in ***Scheme 3.19***. The enzyme facilitates hydrolysis of the N'-acyl moiety to give compound **(74)**, which then decomposes via 2 concurrent pathways to 4-nitroaniline **(92)** (*Path 1, **Scheme 3.19***) or N-(4-aminobenzyl)-4-nitroaniline **(99)** (*Path 2, **Scheme 3.19***).



Scheme 3.19 *Decomposition mechanism of N'-acetyl carbamate (26) with acylarylamidase*

3.5 Synthesis and reactions of model carbamates

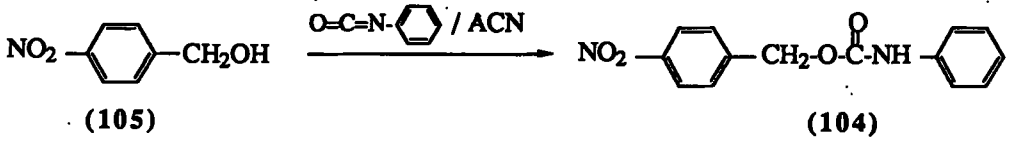
3.5.1 Synthesis and hydrogenolysis of 4-nitrobenzyl *N*-phenyl carbamate (104)

In order to clarify the behaviour of compounds possessing both an aromatic 4-nitro group and a carbamate function under catalytic hydrogenolysis, a model compound 4-nitrobenzyl *N*-phenyl carbamate **(104)** similar to **(75)** but lacking a protected 4-amino group was synthesised and examined.

3.5.1.1 Synthesis

The synthesis of **(104)** followed the procedure previously described for condensation of a benzyl alcohol with an aryl isocyanate. The 4-nitrobenzyl alcohol **(105)** in ACN was reacted with phenylisocyanate for 48h at room temperature (***Scheme 3.20***). After vacuum evaporation of the solvent and extraction into DCM, the compound was purified by silica flash chromatography followed by silica column chromatography using ether/n-hexane (1:1,v/v) as eluent. The impurities removed by the purification included the trimer of phenylisocyanate **(83)** and N,N-diphenyl urea **(84)**.

The pure compound obtained in 71% yield gave m.p. 146-148°C and a satisfactory elemental analysis. It was identified by MS(EI) which gave m/z=272 (M^+), and by ^{1}H-NMR which showed a singlet (2H) at δ=5.2 ppm, attributed to the benzylic CH_2 group, a multiplet plus an AB quartet (J=8.8 Hz) centred at 7.3 and 7.7 ppm (aromatic protons) and integrating to 5H and 4H, respectively, and an exchangeable, broad singlet (1H) at 6.8 ppm, corresponding to the NH proton. The IR spectrum shows a strong absorption at 1740 cm^{-1} confirming the presence of a carbonyl carbamate function.



(105) **(104)**

Scheme 3.20 *Synthesis of* ***(104)***

3.5.1.2 Hydrogenolysis

Compound **(104)** was dissolved in ethyl acetate and stirred under hydrogen with Pd/C catalyst at room temperature as for the hydrogenolysis of compound **(75)**. The reaction was monitored by GC using a BP20 column against authentic 4-toluidine **(89)**, aniline **(106)**, 4-nitrotoluene **(107)** and 4-nitrobenzyl alcohol **(105)** (Experimental 5.4.8.1.2). Aliquots of the reaction mixture were assayed at regular intervals:

a) At t=0 min, only the substrate **(104)** (Rf=66.12 min), and a small peak at Rf=45.38 min [same Rf as 4-nitrobenzyl alcohol **(105)**] were detectable.

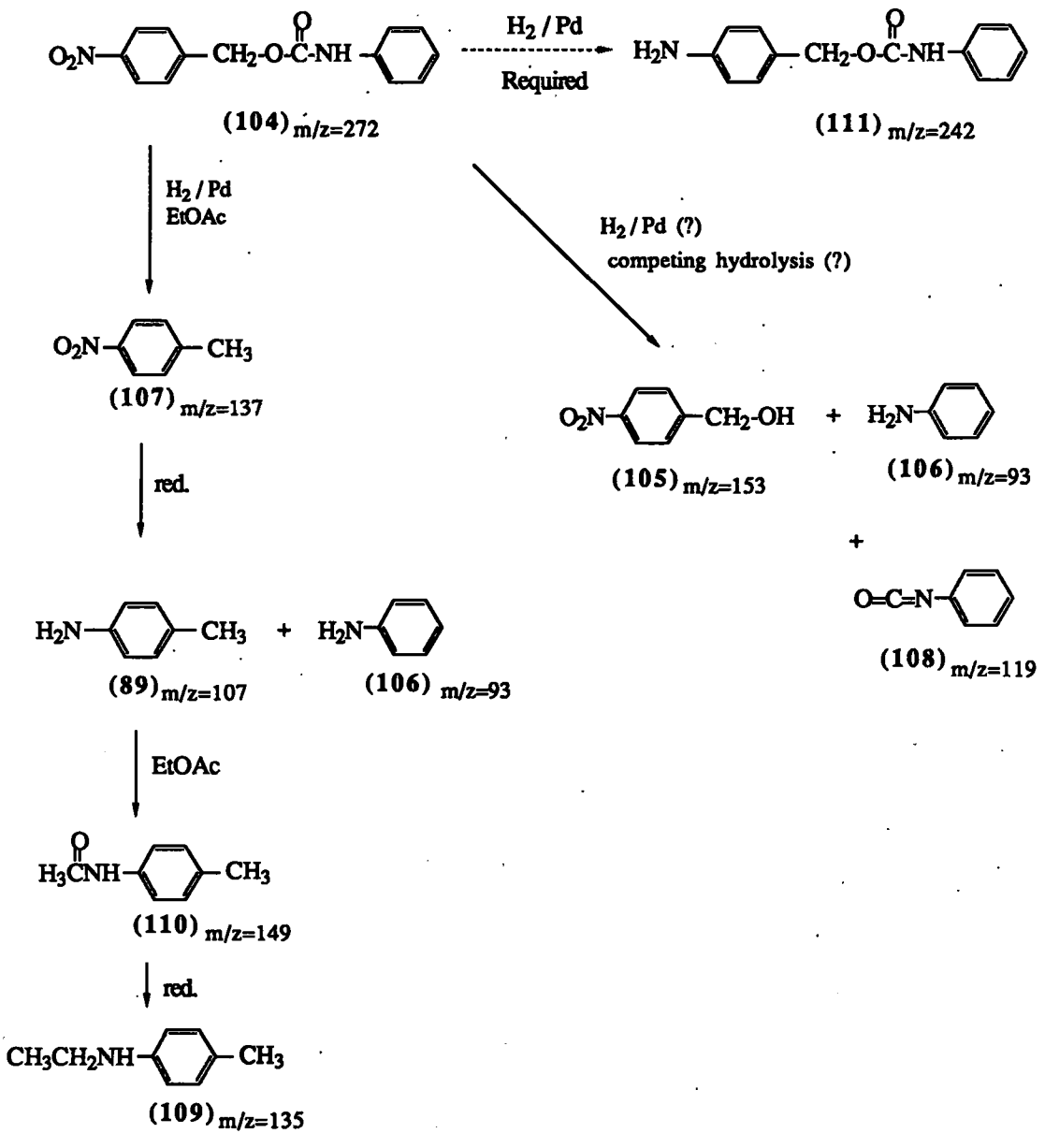
b) After 15 min, two new compounds with Rf=11.79 min (same Rf as aniline **(106)**) and Rf=12.66 min [same Rf as 4-toluidine **(89)**] plus an increased amount of 4-nitrobenzyl alcohol **(105)** were evident. The substrate **(104)** (Rf=66.12 min) had decreased to less than 10% of its original concentration.

c) After 90 min, the chromatogram showed no differences from that at t=15 min except for the complete disappearance of the substrate **(104)**.

Further aliquots injected after 5h and 48h of reaction showed the presence of 4-nitrobenzyl alcohol **(105)**, aniline **(106)** and 4-toluidine **(89)** plus a few new, unidentified small peaks.

An aliquot of the reaction solution after 48h, was also analysed by GC-MS using a BP20 column to confirm the major products. The compounds identified included phenylisocyanate **(108)** (Rf=2.4 min, m/z=119), aniline **(106)** (Rf=4.3 min, m/z=93), *N*-ethyl-4-methylaniline **(109)** (Rf=4.6 min, m/z=135), 4-toluidine **(89)** (Rf=4.8 min, m/z=107), 4-methylacetanilide **(110)** (Rf=7.7 min, m/z=149), 4-nitrobenzyl alcohol **(105)** (Rf=9.6 min, m/z=153), together with 3 other compounds at Rf=5.1, 5.6 and 6.2 min, which were not identified.

The major products of hydrogenolysis were 4-toluidine **(89)** and aniline **(106)**. It was not possible to detect at any stage of the reaction aryl carbamate **(111)** resulting from simple reduction of the 4-nitro group or 4-nitrotoluene **(107)**. These results, summarized in *Scheme 3.21*, suggest a simultaneous hydrogenolysis of the carbamate and the nitro groups.

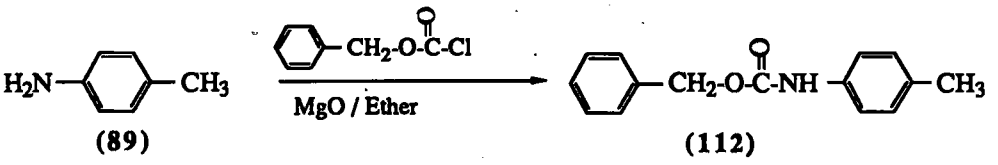


Scheme 3.21 Hydrogenolysis of (104) in H_2/Pd at 25°C

3.5.2 Synthesis of Benzyl *N*-(4-methylphenyl) carbamate (112)

The aryl carbamate (112) was synthezised to assist in identifying the hydrogenolysis and hydrolysis products of arylcarbamates (75) and (76). It was never found, however, among the hydrogenolysis and hydrolysis products of carbamates (75) and (76).

Synthesis of (112) involved condensation of 4-toluidine (89) with benzyl chloroformate at 0°C in the presence of MgO (*Scheme* 3.22), following the procedure previously described for the synthesis of (85).



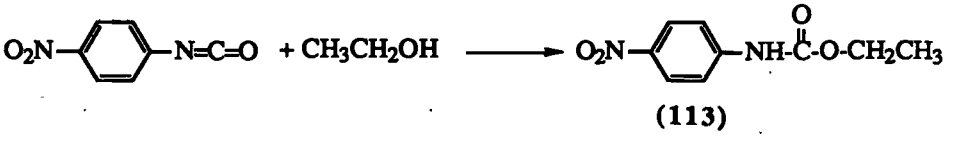
Scheme 3.22 Synthesis of carbamate (112)

The reaction was followed by silica tlc (ether/n-hexane (2:1, v/v). Compound **(112)** was obtained in 89% yield after silica column chromatography using DCM as eluent, and gave m.p. 78-79°C. It was characterised spectroscopically by MS(FAB$^+$) m/z=242 (MH^+) and by MS(FAB$^-$) which showed m/z=240 ($M\text{-}H^+$). The ^{1}H-NMR ($CDCl_3$) spectrum showed two singlets at δ=2.2 (3H) and 5.1 ppm (2H) attributed to the 4-CH_3 and benzylic CH_2 groups, respectively; an AB quartet (4H, J=9.2 Hz), centred at 7.1 ppm and a multiplet (5H) centred at 7.3 ppm, both attributed to the aromatic protons. The IR spectrum showed a strong absorption at 1725 cm^{-1} due to the carbamate carbonyl group.

3.5.3 Synthesis and hydrolysis of ethyl *N*-(4-nitrophenyl) carbamate (113)

3.5.3.1 Synthesis

Synthesis of ethyl *N*-(4-nitrophenyl) carbamate **(113)** involved reaction of 4-nitrophenyl isocyanate with ethanol at 40°C for 1h (***Scheme 3.23***). It was obtained impure in 98% yield, and purified by silica flash chromatography using DCM as eluent. The pure compound gave m.p. 134-135°C. It was characterized by MS(EI) giving m/z=210 (M^+) and ^{1}H-NMR ($CDCl_3$) showing a triplet (3H) at δ=1.3 ppm and a quartet (2H) centred at 4.2 ppm for the ethyl ester group; an AB quartet (4H, J=9.0 Hz) centred at 7.9 ppm for the aromatic protons; and an exchangeable, broad, singlet (1H) at 7.3 ppm due to NH. The IR spectrum showed a strong absorption at 1743 cm^{-1} due to the carbamate carbonyl group.



Scheme 3.23 Synthesis of carbamate (113)

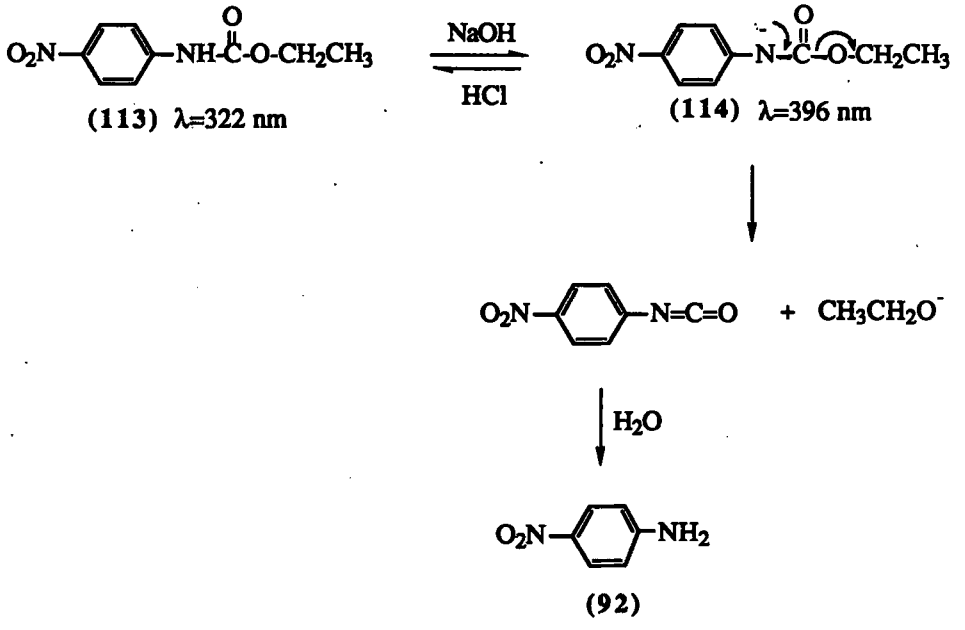
3.5.3.2 Hydrolysis in aqueous/DMSO (9:1,v/v) at 37°C and different pH

Compound (113) in DMSO (10 µl, 14.5 mM) was injected into a thermostatted cuvette at 37°C containing aqueous DMSO (9:1, v/v) at either pH 2, 7 or 12 (adjusted with either HCl or NaOH). The concentration of (113) in the cuvette was 4.8×10^{-5} M. UV spectra were recorded between 250-500 nm immediately, and at timed 20 min intervals.

The reactions at pH 2 and 7 gave λ_{max}=322 nm, which did not vary in intensity or position over 60 min.

The reaction at pH 12 gave λ_{max}=396 and 328 nm at time zero, and the spectrum transformed steadily to λ_{max}=368 nm without a well-defined isosbestic point. These results are shown as *Figure 3.8*.

In a separate experiment involving addition of either acid or base to change the pH as appropriate, it was shown that the spectral differences at time zero relate to prototropic equilibria (involving (113) and the related anion (114)). Further, that the time dependent spectral differences at pH 12 relate to base-catalysed hydrolysis. These transformations are outlined in *Scheme 3.24*.



Scheme 3.24 Equilibrium involving compound (113) in aqueous/DMSO (9:1, v/v) and different pH

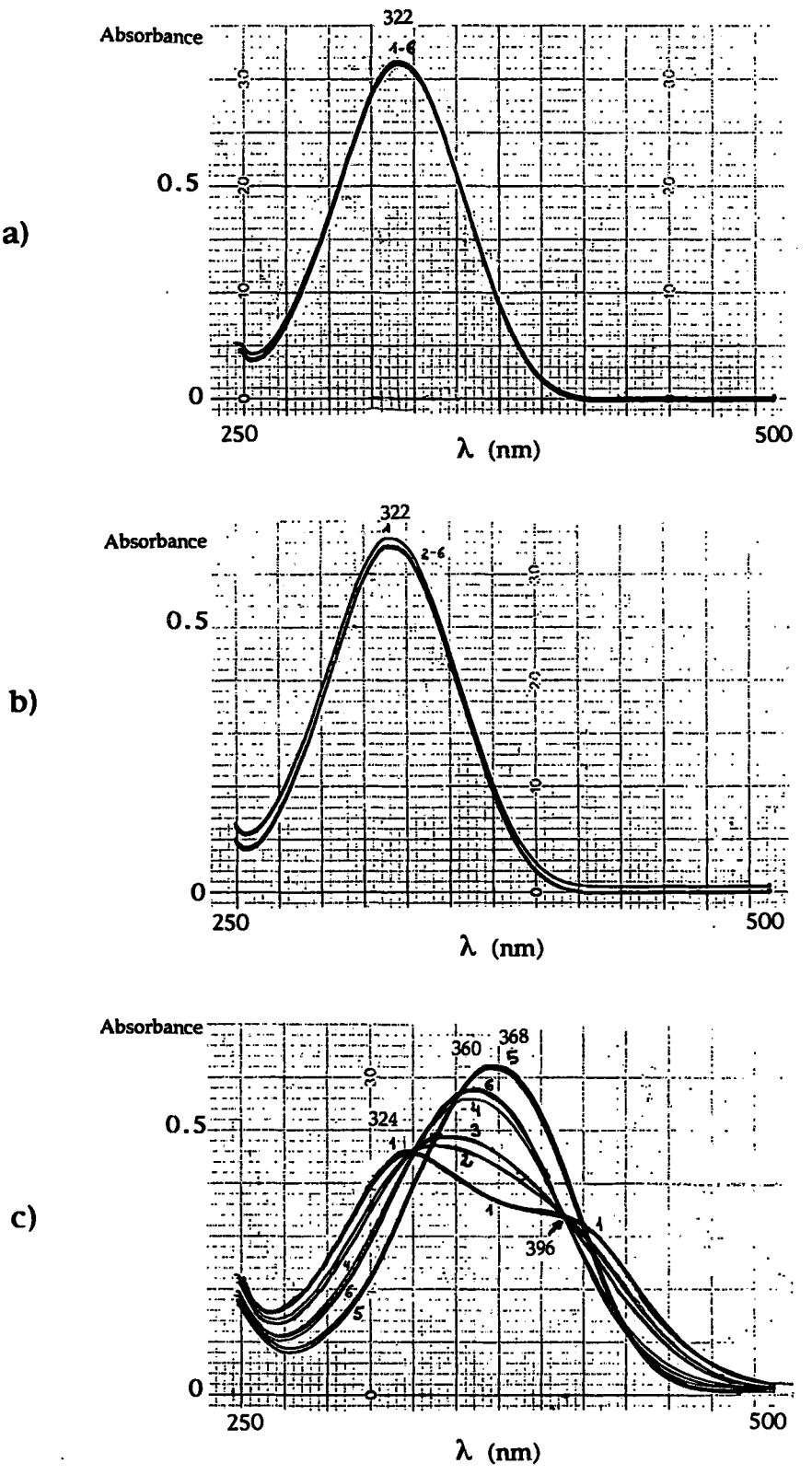


Figure 3.8 Hydrolysis of (113) [4.8x10^{-5}M] in aqueous/DMSO (9:1,v/v) at 37°C and a) pH 2, b) pH 7, c) pH 12

Chapter 4

Summary and Discussion

The project was aimed at the synthesis of aryl N-mustard compounds and evaluation of their suitability as prodrugs for ADEPT (antibody directed enzyme prodrug therapy) against human cancers. The choice of aryl-N-mustard prodrugs resulted from discussions with the Cancer Research Campain (especially via Professor K.D. Bagshawe and his colleagues at Charing Cross Hospital) and Cell-Tech PLC who had both an intellectual and financial interest in the project. Another consequence of these discussions was the selection of N-acetyl protected prodrugs for use with antibody-conjugated *N*-acylarylamidase enzymes. A suitable non-mammalian enzyme conjugated with an antibody specific for choriocarcinoma cells was available from parallel investigations by Charing Cross Hospital and the PHLS at Porton Down.

It was foreseen that suitable prodrugs would have to meet 4 important criteria:

1) Be excellent substrates for the *N*-acylarylamidese enzyme

2) Be highly stable under physiological conditions (ie. pH 6-8 and 37°C)

3) Be unreactive towards cellular substrates in their protected (prodrug) configuration

4) Be highly reactive towards cellular substrates in their deprotected (drug) configuration.

Criteria 3) and 4) are best referred to as a high differential reactivity between the prodrug and drug configurations. Assessment of this parameter was referred to colleagues at the Charing Cross Hospital using cultured cell-lines *in vitro* (see Appendix). The following mainly concerns chemical aspects of the project carried out at The Open University.

4.1 *N*-Acylaminoaryl and related N-mustards

4.1.1 Synthesis

Several compounds were successfully synthesised by adaptations of literature procedures and then fully characterised. In general, the 4-*N*-acylamino compounds were relatively stable and isolable; the parent 4-amino compounds, however, were very labile and difficult to obtain in a highly purified state.

4.1.2 Stability and decomposition

Although aryl-*N*-mustards are relatively well-known compounds, surprisingly little quantitative information is available on their stability and reactivity. This partly relates to their low solubility in aqueous media and to priorities given to the study of *N*-2-chloroethyl compounds which have already found application as chemotherapeutic agents.

a) A series of unsubstituted aniline mustards were successfully synthesised to investigate the effect of the *N*-ethyl 2-substituent on stability and reactivity. These reactions were followed by pH titration and confirmed by HPLC. The effect of leaving group structure on reactivity towards water (hydrolysis) is summarised in ***Table 2.1***. Although there is a parallel between the rates of hydrolysis and both the nucleofugacity [ie. leaving group constant L defined by equation 1.13] for Y^- and the acidity (pKa) of HY [138] on the N-ethyl-2-substituent a good correlation is not evident. The N-2-mesylethyl compounds proved to be the most reactive followed by N-2-chloro compounds. The N-2-nitro, acetyl and thiocyanate compounds are unreactive and probably unsuitable for further study.

b) The 4-substituted aryl-*N*-mustards [H **(5)**, NH_2 **(10)**, $NHCOCH_3$ **(11)**, $NHCOCF_3$ **(12)**] of the bis-*N*-2-chloroethyl series were successively synthesised by adaptation of literature procedures. Their stability towards H_2O (hydrolysis) was investigated at pH 2-12/13 and 37°C by various methods (HPLC, ion-exchange chromatography and UV-visible spectrophotometry). HPLC proved the most useful allowing measurement of substrate loss as well as formation of intermediates and products and the evaluation of sequential decomposition steps.

Table 4.1 summarises the *pseudo* first-order rate constants for the hydrolysis of mustards *N,N*-bis(2-chloroethyl)aniline **(5)**, *N,N*-bis(2-chloroethyl)-4-amino-aniline **(10)**, *N*-[4-[*N'*,*N*'-bis(2-chloroethyl)amino]phenyl]acetamide **(11)** and *N*-[4-[*N'*,*N*'-bis-(2-chloroethyl)amino]phenyl]trifluoroacetamide **(12)** in aqueous DMSO at 37°C under different conditions together with information on the assay method used. The rates at pH 2 are slower than those at higher pH, probably related to protonation of the mustard N-atom.

The rate constants for mustard hydrolysis obtained by different assay methods agree reasonably well. Any differences probably reflect the effect of DMSO in lowering the dielectric constant of the medium. For some methods, the rate constants for both of the sequential decomposition steps (k_1 and k_2) were evaluated [eg. HPLC assay at pH 2 (using enzfitter programe) and ion exchange chromatography assay (using time ratio method)]. The conclusion that $k_1 \cong k_2$ is not too unexpected because of the similarity of the two steps. This result agrees with literature data for the hydrolysis of 4-anilinoquinolinum aniline mustards.[117]

Values of k_1 for the hydrolysis of mustards **(5)**, **(10)**, **(11)** and **(12)** in 3% DMSO phosphate buffer (10^{-3}M) pH 7.4 correlate with Hammett σ parameter (*Figure 4.1*). The fit of this plot ($\log k^X/k^H$ *versus* σ) is reasonable for only 3 data points and the low value of $\rho = -2.27$ is consistent with an Sn2 (Sn_i) displacement process where the *N*-lone pair electrons displace the leaving group to generate an aziridinium ion intermediate as outlined in *Scheme 4.1*. Comparison with the $\rho = -2.73$ for the ionisation of $ArNH_3^+$ in water at 25°C [186] suggests that positive charge development of 80% is localised on the mustard *N*-atom in the transition state. Further, the present results agree reasonably well with $\rho = -1.84$ obtained by Ross *et al.* [41] for the hydrolysis of other aniline mustards.

The low differential reactivity between CH_3CONH or CF_3CONH and NH_2 compounds (factors of 15 and 41, respectively) is too low for good prodrug/drug combination as shown by cell-line studies in the Appendix. This suggests that factors other than chemical deactivation of the 4-amino substituent of mustards (eg. differential transport) are necessary to obtain useful prodrug/drug combinations.

Table 4.1

Rate constants obtained by different assay methods for the hydrolysis of mustards (5), (10), (11) and (12) in aqueous DMSO at 37°C under different conditions

Compound	3% (v/v) DMSO phosphate buffer (10^{-3}M) pH 7.4			
	HPLC [a]		Ion exchange chromatography [b]	
	$10^4 k_1$ s^{-1}	k_1^X/k_1^H	$10^4 k_1$ s^{-1}	$10^4 k_2$ s^{-1}
(5)	1.10	1	2.56	1.79
(10)	17.35	15.8	26.6	16
(11)	1.13	1.03	2.9	1.6
(12)	0.42	0.38	0.86	0.6

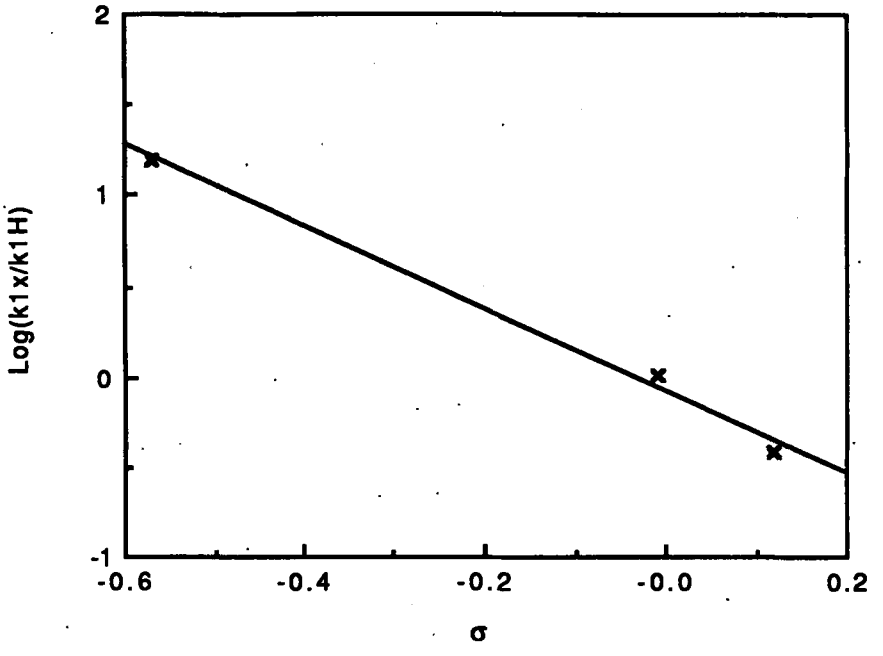
	30% (v/v) aqueous DMSO		
	HPLC		
	pH 2	pH 12	pH 13
	$10^4 k_1$ s^{-1} / 10^4 k_1 and k_2 s^{-1} [c]	$10^4 k_1$ s^{-1}	$10^4 k_1$ s^{-1}
(11)	0.265 / 0.29 ; 0.86	0.453	0.80
(12)	0.098 / 0.044 ; 1.04	3.25	5.05

	1% (v/v) aqueous DMSO	
	UV/Visible	
	pH 2	pH 12
	$10^4 k_0 s^{-1}$	$10^4 k_0$ s^{-1}
(10)	0.26	2.3
(11)	0.96	-
(12)	0.405	5.8

a) rate of disappearance of mustard starting material

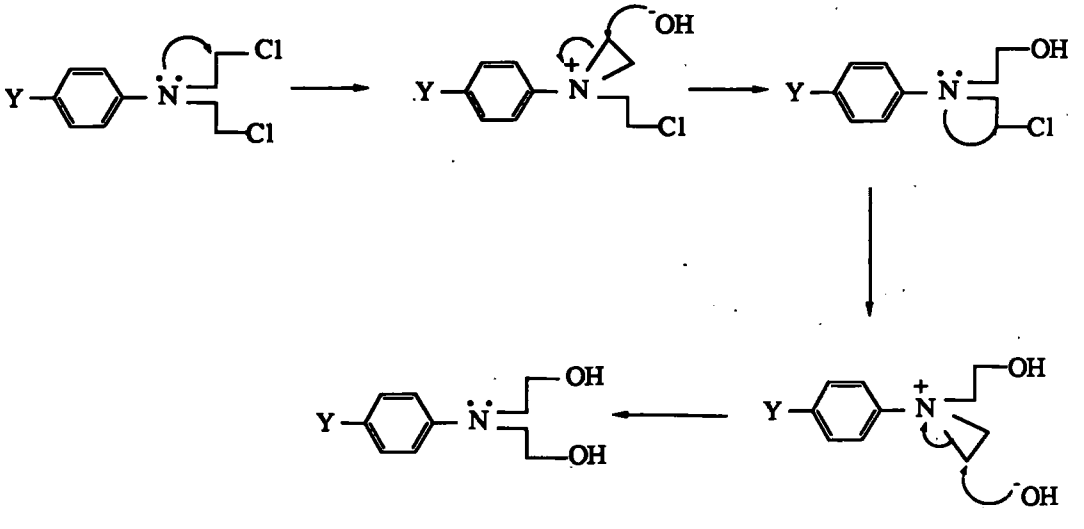
b) rate of elimination of Cl^-; time ratio method for calculating k_1 and k_2

c) k_1 and k_2 calculated by enzfitter programe



(values of σ: NH_2= - 0.57, CH_3CONH= - 0.01, CF_3CONH= 0.12) [187]

Figure 4.1 *Hammet plot for the hydrolysis of (5), (10), (11) and (12) in phosphate buffer (10^{-3}M) in 3% (v/v) DMSO at pH 7.4 and 37℃*



Scheme 4.1 *Two step decomposition pathway for mustard hydrolysis*

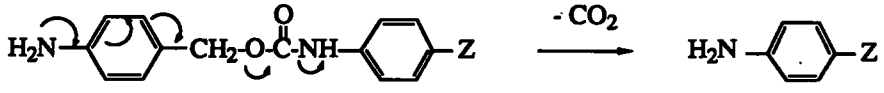
The product analyses show that at high pH, cyclisation occurs to form morpholine derivatives, as first observed in 1973.[188] At physiological pH, however, this cyclisation is unimportant.

To further understand the mechanism of mustard hydrolyses, the behaviour of mustards **(11)** and **(12)** in aqueous DMSO (7:3,v/v) at 37°C and different pHs (pH 2, 7, 12 and in some cases pH 13) was investigated. The reactions were monitored and the final products identified and quantified by HPLC. Identification of isolated products was confirmed by MS(FAB) and (EI), GC and GC-MS. The HPLC studies revealed a steady decrease with time of the aryl-N-mustard substrate concomitant with the formation of an intermediate half-hydrolysed mustard, which then decreased with formation of the final products. In some cases, the final products proved unstable and reacted further. This information is entirely consistant with the mechanism outlined in ***Scheme 4.1***.

The hydrolyses of mustards **(10)**, **(11)** and **(12)** in aqueous DMSO (1%, v/v) at 37°C and at pH 2, 7.4 and 12, were also followed by UV/visible spectrophotometry. The similarity of the absorbances throughout the entire reaction for all the aryl N-mustards at pH 7 and for mustard **(11)** at pH 2 prohibited evaluation of rate constants.

4.2 Carbamate drug/prodrug combinations

The main aim here was to establish that after removal of protecting groups, 4-aminobenzylcarbamate structures **(72)** spontaneously decompose to give 4-aminoaryl products **(73)** according to ***Scheme 4.2***.



(72) Reactive carbamate **(73)** Drug

Scheme 4.2 *Spontaneous decomposition of 4-aminobenzylcarbamate strutcure into 4-aminoaryl products*

The synthesis of the carbamate structures was accomplished by adaptation of literature procedures. The key problem was the protection of the 4'-amino group. Out of several protection groups tested (CBZ, trityl, Ac and *t*-BOC) the *t*-BOC derivative was found

to be most suitable. Conditions were found for the removal of this group without damaging the central benzyloxycarbonyl bond in the molecule. Synthesis of both the *t*-BOC protected and unprotected compounds was achieved for model compounds without an N-mustard moiety.

The main problems involved with the use of the CBZ protection relate to its removal by hydrogenolysis, which preferentially removed the central carbamate moiety instead of the carbamate group in the CBZ protection. With the trityl protection the problems relate with the lability of this group which did not allow to obtain a pure compound.

Decomposition (hydrolysis) of the 4-*N*'-acyl model substrate to a 4-amino aryl product was briefly examined at pH 2-13 at 25°C. It was possible to show that at pH 7 the 4-*N*'-acetyl compound is stable whereas the 4-NH_2 compound is very unstable to give products of spontaneous decomposition which include a 4-aminoaryl product (73), a *N*-(4'-aminobenzyl)-aryl product (resulting from recombination of a pair of ions generated) and a polymeric structure. It was then shown that 4-aminobenzylcarbamate prodrugs should spontaneously decompose to an active drug on removal its protecting group. The 4'-*N*-acetyl compound proved to be a good substrate for an acylarylamidase enzyme which suggests that after introduction of the N-mustard moiety, these structures could be suitable prodrugs for ADEPT.

Chapter 5

Experimental

5.1 General Instrumentation

Infra-red spectra were recorded as either thin films, nujol mulls or KBr discs, using a Perkin-Elmer 1410 spectrophotometer against a polystyrene standard.

Ultra-violet/visible spectra were run on either Cecil CE 5501, Kontron Uvikon 810P or LKB 4050 spectrophotometers, calibrated with a holmium filter.

^{1}H Nuclear magnetic resonance spectra were provided by the Departmental service and recorded either on a Jeol FX90Q FT or a Jeol JNM-FX400. The internal standard for organic solvents was tetramethylsilane.

Mass spectra were provided by the Departmental service and obtained using a VG 20-250 instrument. Spectra provided were either electron impact, fast atom bombardment (with xenon as the ionising gas) or gc modulated (using a HP 4840 gc).

Microanalyses were supplied by Medac Ltd at Brunel University.

Melting points were measured on an Electrothermal digital melting point apparatus and are uncorrected.

pH measurements were made on a Radiometer PHM 82 pH meter, equipped with a titrator TTT 80 and autoburette ABU 80, using Radiometer G2040C glass and K4040 calomel electrodes, calibrated with BDH standard buffers.

HPLC analysis were run on a ternary gradient liquid chromatography, Varian model 5060, equiped with a Varian 9060 diode array detector, coupled to a Physics chromojet computing integrator.

GC analysis were obtained with a Carlo Erba ,Vega Series II 6180 instrument.

Ion exchange chromatography was performed on a Milton Roy 3000 solvent delivery system equipped with a LDC/Milton Roy conductivity monitor III.

A mathematical package, Enzeffiter-(EGA-version 1.03) by Elsevier-Biosoft,[159] was used to analyse some kinetic data.

5.2 Reagents and Reactants

Reagents used for kinetic studies and buffer solutions, NaCl, $NaClO_4$, $Na_2B_4O_7$ and 1.0M NaOH (AVS) and HCl, tris(hydroxymethyl aminomethane), were Analar grade, from BDH and were used without further purification.

Solvents and buffers components for HPLC (ACN, DMSO, phosphoric acid, potassium dihydrogen orthophosphate, acetic acid, sodium acetate and phtalic acid) were either Analar or HPLC grade and were used as supplied.

The following reagents for syntheses were laboratory grade from commercial suppliers and were used as supplied: acetamide, 4-acetamidophenol, acetanilide (97%), acetic anhydride, 4-aminoacetanilide, 4-aminoaniline, 4-aminobenzotrifluoride, 4-aminobenzyl alcohol, 4-aminophenol, *N*-(4-aminophenyl)acetamide, benzhydrol, benzyl alcohol, butylithium (1.6M) in hexane, di-tert-butyl dicarbonate, 4-chlorobenzylalcohol (99%), 4-chloroacetanilide, 4-chloroaniline (98%), bis(2-chloroethyl)amine hydrochloride (98%), 1-chloro-4-nitrobenzene, diethanolamine, ethanolamine, ethylene oxide, glacial acetic acid, methanesulfonyl chloride, 4-methoxybenzamide (97%), 2-methyl-2-propanol, 4'-methylacetanilide, morpholine, 4-morpholinoaniline (98%), 4-nitroaniline, 4-nitroacetanilide, 4-nitrobenzamide (98%), 4-nitrobenzylalcohol, 4-nitrotoluidine, 10% palladium on charcoal, phenylisocyanate, 4-phenylmorpholine (98%), picric acid (1.0 wt. % in water), sodium acetate, sodium nitrite, sodium thiocyanate, sodium tetrafluoroborate (98%), toluene-4-sulphonyl chloride, p-toluidine, 4-trifluoromethylphenylisocyanate, triphenylmethanol (97%), triphenylmethylchloride, α,α,α-trifluoro-p-tolylisocyanate, trifluoroacetic anhydride, trifluoroacetic acid, α,α,α-trifluoroacetamide, and gaseous hydrogen chloride (Fisons).

Aniline, acetyl chloride, 4-nitrophenyl isocyanate, pyridine and triethylamine were purified by standard methods.[189]

N,N-Bis(2-hydroxyethyl)-4-amino-aniline sulphate monohydrate was purified by the method described in Section 5.3.21, and 2-mercaptoethanesulfonic acid sodium salt (98%) was converted in the acid form according to procedure described in Section 5.3.32.

The solvents acetone, acetonitrile, benzene, dichloromethane, ethanol, 2-ethoxyethanol, ethylacetate, methanol, tetrahydrofuran and toluene were also purified by standard procedures.[189]

Isotopically labelled compounds (MSD Isotopes) were used as supplied.

Thin-layer chromatography was carried out using silica-gel UV_{254} (0.25mm) plates and the compounds visualized using UV light. Column chromatography was carried out using silica-gel, 60°A, obtained from usual suppliers.

Acylarylamidase enzyme (E.C. N° 3.5.1.13) was a gift from Dr. Roger Sherwood, Division of Biotechnology, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, UK.

5.3 Synthesis of substrates

5.3.1 *N,N*-Bis(2-hydroxyethyl)aniline (37)

Following a literature procedure,[41,43] aniline (14 g, 150 mmol) and ethylene oxide (15 g, 340mmol) were heated at 150°C, for 20 h, in a Carius tube. Due to the volatility of ethylene oxide, the Carius tube containing the aniline was cooled in ice (0 to -5°C) before adding the ethylene oxide, and after sealing, was heated in suitably screened oven. The white solid product was recrystallised 5 times from benzene to give white plates of *N,N*-bis-(2-hydroxyethyl)aniline **(37)**, yield 19.3 g (71%); m.p. 55.4-56°C (lit.[43] m.p. 55°C); ν_{max} (nujol) 3350 (OH), 1600 (ArH), 1500 (ArH), 1330, 750 cm^{-1}; δ^1H ($(CD_3)_2CO$) 3.6 and 3.8 (8H, dt, CH_2), 4.7 (2H, br s, *OH*), 6.7-7.2 centred at 7.0 ppm (5H, m, Ar*H*).

5.3.2 *N,N*-Bis(2-methanesulphonyloxyethyl)aniline (38) and *N,N*-Bis(2-methanesulphonyloxyethyl)aniline hydrochloride salt (43)

Methanesulphonyl chloride (2.4 cm^3, 3 mmol) was added with vigorous stirring, to a solution of *N,N*-bis(2-hydroxyethyl)aniline (2.4 g, 13 mmol) in dry pyridine (15 cm^3) at -5°C, and the mixture stirred at this temperature for 30 min. Water (1.3 cm^3) was then added in three portions, with stirring, at *ca.* 5°C. The mixture was quickly diluted with ice-cold aqueous sulphuric acid (4.5 M; 36 cm^3), immediately extracted into chloroform (20 cm^3), and the extract vacuum evaporated to give *N,N*-bis(2-methanesulphonyloxyethyl)aniline **(38)** as a syrupy product, yield 0.57 g (56%). A portion of this syrup was purified by silica column chromatography using DCM as eluent, ν_{max} (thin film) 3005 (NH), 2910, 1600, 1500, 1350 (OSO_2CH_3), 1175 (OSO_2CH_3), 750 and 700 (ArH) cm^{-1}; δ^1H ($CDCl_3$) 2.9 (6H, s, OSO_2CH_3), 3.7 (4H, t, J=6.8Hz, NCH_2), 4.3 (4H, t, J=6.8Hz, CH_2OSO_2), 6.7-7.4, centred at 7.05 ppm (5H,

m, Ar*H*); m/z (EI) 337 (18%, M^+), 228 (100, M^+ - $CH_2OSO_2CH_3$), 132 (43), 123 (72, $^+CH_2CH_2OSO_2CH_3$), 105 (38), 104 (38), 91 (17, $C_7H_7^+$), 79 (48, $^+SO_2CH_3$).

The *N,N*-bis(2-methanesulphonyloxyethyl)aniline **(38)** (1.0 g) was converted into the hydrochloride salt by dissolving in chloroform (4 cm^3) and passing dry, gaseous hydrogen chloride through the solution, until saturated. The solution was then refrigerated overnight. The precipitated crystals were filtered, washed with cold chloroform (without exposure to the air) until practically colourless, and pressed dry. The crystals were then suspended in cold acetone (precooled in dry ice-chloroform), refiltered and dried, giving colourless crystals. Yield 0.83 g (75%); m.p. 77-82°C (lit.[140] m.p. 98-105°C); ν_{max} (nujol) 3400 (NH_3^+), 1350 (OSO_2CH_3), 1175 (OSO_2CH_3), 1040, 720 cm^{-1}; δ^1H ($(CD_3)_2SO$) 3.2 (6H, s, CH_3), 3.75 (4H, t, J=6Hz, NCH_2), 4.35 (4H, t, J=6Hz, OCH_2), 6.7-7.3 centred at 7.0 ppm (6H, m, Ar*H* and N*H*) ; m/z (FAB^-) 372 (0.4%, $M\text{-}H^+$), 95 (100, $CH_3SO_2O^-$), 35 (14, Cl^-).

5.3.3 *N,N*-Bis(2-acetoxyethyl)aniline (39)

N,N-Bis(2-hydroxyethyl) aniline **(37)** (0.5 g, 2.8 mmol) was suspended in pyridine (5 cm^3), at room temperature. Acetyl chloride (0.98 cm^3, 14 mmol) was added slowly and the reaction allowed to proceed for 3h at room temperature. The disappearance of the starting material was followed by silica tlc (DCM). The pyridine was removed under vacuum and low temperature, and the impure residue, yield 0.5 g (69%), column chromatographed on silica using ether/DCM (1:1, v/v) as eluent. The product was obtained after evaporation of the solvent as an yellow orange oil, 0.37 g (51%) (Found: C, 63.2; H, 7.3; N, 5.2. $C_{14}H_{19}NO_4$ requires: C, 63.4; H, 7.2; N, 5.3%); ν_{max} (nujol) 1670 (C=O), 1600, 1500, (CH_2OCO), 1380 ($OCOCH_3$), 745 and 690 cm^{-1} (ArH); δ^1H ($(CD_3)_2CO$) 2.0 (6H, s, $OCOCH_3$), 3.7 (4H, t, J=7.5Hz, NCH_2), 4.3 (4H, t, J=7.5Hz, OCH_2), 6.7-7.3, centred at 7.05 ppm (5H, m, Ar*H*); m/z (FAB^+) 266 (18%, MH^+), 206 (8, M^+- $OCOCH_3$), 192 (25, M^+- CH_2OCOCH_3), 146 (4), 132 (6), 120 (6), 104 (8), 91 (7, $C_7H_7^+$), 87 (100, $^+C_2H_4OCOCH_3$), 43 (44, CH_3CO^+).

5.3.4 *N,N*-Bis(2-ethyltoluene-4-sulphonate)aniline (44)

Following a literature procedure,[140,146] *N,N*-bis(2-hydroxyethyl)aniline (2.0 g, 11 mmol) was dissolved in pyridine (22 cm^3), and toluene-4-sulphonylchloride (9.2 g, 48 mmol) was added in two aliquots, at 1h intervals, whilst the mixture was stirred at 0°C for 2h and for a further 1h at room temperature. The mixture was then cooled and treated dropwise with sufficient water to decompose the excess sulphonyl chloride. More water was added (60 cm^3)

and the resulting suspension was stirred overnight to give a pale yellow solid, which was then filtered off, washed with water (60 cm^3), and dried to give crude *N,N*-bis(2-ethyltoluene-4-sulphonate)aniline, yield 4.4 g (82%). The solid was further recrystalised from ethanol to give white needles. Yield 4.1 g (75%); m.p. 88-89°C (lit.[140] m.p. 90-91°C); ν_{max} (nujol) 1600 (ArH), 1500 (ArH), 1360, 1100, 800, 750 cm^{-1}; δ^1H ($(CD_3)_2CO$) 2.4 (6H, s, CH_3), 3.6 and 4.1 (8H, dt, CH_2), 6.4-7.2 centred at 6.8 (5H, m, Ar*H*), 7.3-7.8 centred at 7.5 ppm (8H, ABq, J=9.1Hz, Ar*H*); m/z (EI) 489 (10%, M^+), 304 (89, M^+- CH_2OTs), 91 (100, $C_7H_7^+$).

5.3.5 *N,N*-Bis(2-chloroethyl)aniline (5)

A solution of *N,N*-bis(2-ethyltoluene-4-sulphonate)aniline (3.4 g, 7 mmol) and anhyd. $CaCl_2$ (2.0 g, 18 mmol) in dry 2-ethoxyethanol (25 cm^3), was stirred at 120°C, for 1.5h and then allowed to cool. The solvent was removed under vacuum and the mustard was extracted into benzene (100 cm^3). The benzene extract was washed with water (100 cm^3) and dried with anhyd. $MgSO_4$. On evaporation of the solvent, *N,N*-bis(2-chloroethyl)aniline was obtained as a syrup, yield 0.9 g (60%). It was recrystallised from toluene/n-hexane (1:1, v/v) giving a solid m.p. 43-45°C (lit.[43] m.p. 45°C); ν_{max} (nujol) 1600 (ArH), 1500 (ArH), 770, 650 cm^{-1}; δ^1H ($(CD_3)_2CO$) 3.7 (8H, s, CH_2), 6.7-7.3 centred at 7.0 ppm (5H, m, Ar*H*); m/z (EI) 217 (11%, M^+), 183 (25, $M^{+\cdot}$ - Cl), 168 (100, $M^{+\cdot}$ - CH_2Cl).

5.3.6 *N,N*-Bis(2-thiocyanoethyl)aniline (40)

A solution of *N,N*-bis(2-ethyltoluene-4-sulphonate)aniline **(44)** (1.24 g, 2.5 mmol) and sodium thiocyanate (0.75 g, 9.3 mmol), in dry 2-ethoxyethanol (13 cm^3), was stirred at 120°C for 3h. The disappearance of the starting material was followed by silica tlc using DCM as eluent. The solvent was removed under vacuum, the product was extracted into toluene (30cm^3), and the toluene extract washed with water (30 cm^3) and dried (anhyd. $MgSO_4$). On evaporation of the solvent, a solid white residue was obtained. This residue was purified by silica column chromatography using DCM as eluent. The fractions containing the product were combined, the solvent removed under vacuum, and the white residue was further purified by 2 recrystallisations from DCM/n-hexane (1:1, v/v), to give white crystals. Yield 0.23 g (49%); m.p. 68-70°C (lit.[147] m.p. 73-74°C); λ_{max} (DMSO) 280 nm; ν_{max} (KBr disc) 3076 (NH), 2153 (CN), 1592 (N≡), 722 (CS), 748 and 690 (ArH); δ^1H ($(CD_3)_2CO$) 3.3 (4H, t, J=8Hz, NCH_2), 3.8 (4H, t, J=8Hz, OCH_2), 6.7-7.3 centred at 7.0 ppm (5H, m, Ar*H*); m/z (FAB^+) 264 (4%, MH^+), 263 (6), 251 (2), 236 (3, M^+- HCN), 223 (3), 205 (21, M^+-SCN), 191 (26,

M^+ - CH_2SCN), 178 (5), 164 (7), 146 (9), 132 (13), 115 (23), 104 (19), 86 (12), 75 (41), 57 (36), 57 (36), 45 (46).

5.3.7 *N,N*-Bis(2-nitratoethyl)aniline (41)

To *N,N*-bis(2-chloroethyl)aniline (5) (0.4 g, 1.85 mmol) in dry ACN (14 cm^3), was added silver nitrate (1.2 g, 7.06 mmol) in dry ACN (2 cm^3). The mixture was left to react under nitrogen, with vigorously stirring, at 40° C for 15h. The reaction was followed by silica tlc using ether as eluent. On completion, the ACN was vacuum evaporated, the residue extracted with ether, and the ether layer washed with water (10 cm^3), then dried over anhyd. $MgSO_4$. The residue obtained after vacuum evaporation of the solvent [yield 0.34 g (66%)] was chromatographed on a silica column using ether/n-hexane (1:2, v/v) as eluent. The product recovered after vacuum evaporation of the solvent, was a yellow oil. Yield 0.21g (41%); λ_{max} (DMSO) 260 nm; ν_{max} (thin film) 2900, 1625, 1600, 1500, 1380, 1280, 1200, 980, 840, 740, 700 cm^{-1}; δ^1H ($(CD_3)_2CO$) 3.8 (4H, t, J=7Hz, NCH_2), 4.75 (4H, t, J=7Hz, OCH_2), 6.7-7.3 centred at 7.0 ppm (5H, m, *Ph*); m/z(EI) 271 (20%, $M^{+}\cdot$), 195 (27, $M^{+}\cdot - \cdot CH_2ONO_2$), 149 (46, $C_6H_5N^+(CH_2)(C_2H_4O\cdot)$), 119 (97, 195 - CH_2ONO_2), 105 (33, $C_6H_5CH_2CH_2^+$), 91 (100, $C_7H_7^+$), 77 (45), 46 (39, NO_2).

5.3.8 *N,N*-Bis(2-hydroxyethyl)-4-nitroso-aniline (50)

N,N-Bis(2-hydroxyethyl)aniline (0.2 g, 1.1 mmol) was dissolved in warm conc. HCl (0.48cm^3, 1.58 mol) and water (0.45 cm^3). The mixture was cooled to 5°C, and a solution of sodium nitrite (0.08 g, 1.2 mmol) in water (0.3 cm^3), was added dropwise, under vigorously stirring, maintaining the the temperature at 5-7°C. After the addition, the mixture was stirred for a further 30min, after which a saturated aqueous solution of $NaHCO_3$ was added dropwise until pH 8 was obtained. The solution was then saturated with sodium perchlorate and extracted with ethyl acetate. The organic extracted was washed with water (2 x 25cm^3) and dried ($MgSO_4$), then purified by silica column chromatography with ethyl acetate as eluent. A tlc showed a pure product. Yield 0.17 g (74%); m.p. 134-136°C (lit.[152] m.p. 137-138°C); ν_{max} (KBr disc) 3350, 1620, 1320, 1120, 1040, 840 cm^{-1}; δ^1H ($(CD_3)_2CO$) 3.0 (4H, s, NCH_2), 3.8 (6H, d, CH_2OH and *OH*), 6.9-7.7 ppm (4H, ABq, J=10.1Hz, Ar*H*); m/z (EI) 210 (21%, M^+), 179 (47, M^+- CH_2OH), 165 (12, M^+- CH_2CH_2OH), 149 (100, M^+- (CH_2OH+NO)), 105 (34, $HN^{+}\cdot (C_2H_4OH)_2$).

5.3.9 *N,N*-Bis(2-methanesulphonyloxyethyl)-4-nitroso-aniline (51)

N,N-Bis(2-methanesulphonyloxyethyl)aniline hydrochloride **(43)** (4.1 g, 11 mmol) was suspended in ACN (20 cm^3) and the solution cooled to -5 to 0 °C. Then, $HClO_4$ (60%; 6.6 cm^3) and water (6 cm^3) was added dropwise, keeping the solution at low temperature. A solution of $NaNO_2$ (1 g, 14 mmol) in water (2 cm^3) was added dropwise over a period of 30min, and the solution was stirred for further 30min. The solution was then neutralized with NaOH to pH 7 (temperature kept below 0°C), stirred for another 1h, and then extracted with DCM (3 x 50 cm^3). On vacuum evaporation of the solvent, a dark green residue was obtained which was purified by silica column chromatography using ethyl acetate as eluent. Yield 2.5 g (62%); m.p. 116-118°C (lit.[7] m.p. 117-118.5°C) (Found: C, 38.7; H, 4.9; N, 7.5. $C_{12}H_{18}N_2O_7S_2$ requires C, 39.3; H, 4.9; N, 7.6 %); ν_{max} (KBr disc) 3030-2930, 1620, 1530, 1380 (NO), 1350, 1175 (OSO_2CH_3), 1120, 980, 840 cm^{-1}; δ^1H ($(CD_3)_2CO$) 3.1 (6H, s, CH_3), 4.1 (4H, t , J=6Hz, NCH_2), 4.6 (4H, t , J=6Hz, OCH_2), 7.1-7.8 ppm (4 H, ABq, J=10.3Hz, Ar*H*); m/z (FAB^+) 367 (2, MH^+).

5.3.10 *N,N*-Bis(2-chloroethyl)-4-nitroso-aniline (52)

Following a literature procedure,[41] *N,N*-bis(2-chloroethyl)aniline (2.0 g, 9.2 mmol) was dissolved in warm conc. HCl (3.9 cm^3) and water (3.7 cm^3). After cooling to 5°C, the mixture was vigorously stirred and a solution of sodium nitrite (0.67 g, 10 mmol) in water (1.85 cm^3) was added dropwise while maintaining the temperature at *ca.* 5-7°C. The mixture was stirred for further 20min and then ether (40 cm^3) and water (40 cm^3) added. The aqueous layer was separated and extracted with ether (2 x 40 cm^3). The ether extracts were combined and dried with anhyd. $MgSO_4$. Vacuum evaporation of the solvent, followed by recrystallisation from ether, gave deep green plates of *N,N*-bis(2-chloroethyl)-4-nitroso-aniline. Yield 1.8 g (79%); m.p. 79-80°C (lit.[41] m.p. 79-80°C); ν_{max} (nujol) 3268 (NH), 1600 (ArH), 1500 (ArH), 1380 (NO), 1100 cm^{-1}; δ^1H ($(CD_3)_2CO$) 4.0 (8H, dt, CH_2), 6.9-7.9 centred at 7.4 ppm (4H, ABq, J=9.0Hz, Ar*H*); m/z (EI) 246 (18%, M^+), 197 (72, M^+- CH_2Cl), 167 (100, M^+ - CH_2ClNO), 63 (50, $^+CH_2CH_2Cl$).

5.3.11 *N,N*-Bis(2-nitratoethyl)-4-nitroso-aniline (53)

N,N-Bis(2-nitratoethyl)aniline **(41)** (0.41 g, 1.5 mmol) was dissolved in water (0.5 cm^3) and ACN (5 cm^3). Then, aqueous $HClO_4$ (60%; 0.7 cm^3) was added at room temperature and, after

cooling to 0-5°C, $NaNO_2$ (0.11 g, 1.6 mmol) in water (0.5 cm^3), was added dropwise, over 20min. The reaction, followed by silica tlc using DCM as eluent, was allowed to proceed for 30min at 0-5°C, after which the ACN was vacuum evaporated and the aqueous residue extracted with ether (15 cm^3), washed with water (10 cm^3), and dried over anhyd. $MgSO_4$. After vacuum evaporation of the solvent, a green solid was obtained. Yield 0.2 g (44%). This was further purified by silica column chromatography, using DCM as eluent. After evaporation of the solvent, the product was obtained as a green crystalline solid. Yield 0.14 g (30%); m.p. 85-87°C (Found: C, 40.2; H, 4.0; N, 18.2. $C_{10}H_{12}N_4O_7$ requires: C, 40.0; H, 4.0; N, 18.6%); ν_{max} (KBr disc) 1640, 1620, 1525, 1380 (NO), 1160, 1130, 900, 860, 830 cm^{-1}; δ^1H ($(CD_3)_2CO$) 4.14 (4H, t, J=6.7Hz, NCH_2), 4.95 (4H, t , J=6.7Hz, OCH_2), 7.0-7.8 centred at 7.45 ppm (4H, ABq, J=10.3Hz, Ar*H*); m/z (FAB^+) 301 (5%, MH^+), 224 (2, MH^+-$MeONO_2$); m/z (EI) 300 (45%, M^+), 224 (10, M^+- CH_2ONO_2), 178 (87, 224 - NO_2), 148 (100, $(ON)C_6H_4CH_2CH_2^+$), 120 (64, $C_6H_5N^{+\cdot}(CH_3)(CH_3)$), 91 (62, $C_7H_7^+$), 90 (66), 77 (48, $C_6H_5^+$), 56 (67), 46 (30, NO_2^+), 30 (53, NO^+).

5.3.12 *N,N*-Bis(2-chloroethyl)-4-amino-aniline (10) and *N,N*-bis(2-chloroethyl)-4-amino-aniline hydrochloride (54)

A solution of *N,N*-bis(2-chloroethyl)-4-nitroso-aniline (52) (0.8 g, 3.2 mmol) in methanol (180 cm^3), containing 15% palladium on charcoal (0.15 g), was stirred under hydrogen, at atmospheric temperature and pressure, until the calculated volume of hydrogen was absorbed and the green colour had disappeared. The solution was filtered and the filtrate added to methanolic HCl (1:1, v/v; 21 cm^3). On evaporation of the methanol, a brown residue was obtained which quickly darkened: it was therefore used immediately in the next stage of the synthesis. The crude yield was 0.7 g (81%). A small amount of the brown residue (0.2 g) was twice recrystallised from methanol/ether (1:1, v/v) to obtain a pure product as almost colourless plates, m.p. 245-258°C (lit.[156] m.p. 250-260°C); ν_{max} 2950 (NH_3^+), 2600, 1620, 1520, 1410, 1370, 820, 740 cm^{-1}; δ^1H ($(CD_3)_2SO$) 3.8 (8H, s, CH_2), 6.8-7.3 centred at 7.0 (4H, ABq, J=9.4Hz, Ar*H*), 10.3 ppm (3H, br s, N*H*); m/z (FAB^+) 233 (10%, MH^+ - HCl).

5.3.13 *N,N*-Bis(2-methanesulphonyloxyethyl)-4-amino-aniline (55) and *N,N*-bis(2-methanesulphonyloxyethyl)-4-amino-aniline methanesulphonate salt (56)

A solution of *N,N*-bis(2-methanesulfonoxyethyl-4-nitroso-aniline (51) (0.56 g, 1.55 mmol) in methanol (190 cm^3), containing 15% palladium-charcoal (0.08 g), was stirred under hydrogen until the calculated amount of gas had been taken up. The light yellow solution was filtered

under nitrogen into methanesulfonic acid (0.25 cm^3, 2.5 eq) in methanol (50 cm^3). After the filtration, the solution was pale green, but on evaporation of the methanol, a brown oily residue (ca. 40% yield) which rapidly darkened, was obtained. Therefore, it was used immediately in the next stage of the synthesis.

5.3.14 *N*-[4-[*N'*,*N'*-Bis(2-chloroethyl)amino]phenyl]acetamide (11)

N,*N*-Bis(2-chloroethyl)-4-amino-aniline hydrochloride (54) (0.44 g, 1.6 mmol) was suspended in DCM (15 cm^3) and shaken with triethylamine (0.56 cm^3, 4.0 mmol). When all the solid had dissolved, the mixture was cooled in ice (0°C), and acetic anhydride (1.5 cm^3, 16 mmol) was added, with vigorous shaking. After 20min, the organic layer was washed with water (15 cm^3) and dried over anhyd. $MgSO_4$. After removing the solvent, the dark residue was crystallized from ether/n-hexane (1:1, v/v). The product was further purified by silica column chromatography using ether/DCM (3:1, v/v) as eluent. After removal of the solvent, the product was twice recrystallised from ether/n-hexane (1:1, v/v). Yield 0.11 g (25%); m.p. 120°C (lit.[41] m.p. 124-126°C) (Found: C, 52.3; H,5.9; N, 9.9. $C_{12}H_{16}Cl_2N_2O$ requires C, 52.4; H,5.9; N, 10.2%); λ_{max} (DMSO) 278nm (logε= 4.4); ν_{max} (KBr disc) 3300 (NH), 1650 (C=O, amide), 1540 (ArH), 1520, 1330, 1180, 1140, 820 cm^{-1} ; δ^1H ($(CD_3)_2CO$) 2.1 (3H, s, CH_3), 3.7 (8H, s, CH_2), 6.7 - 7.5 centred at 7.1 (4H, ABq, J=10.0Hz, Ar*H*), 10.1 ppm (1H, br s, N*H*) ; m/z (EI) 274 (12%, M^+), 225 (93, M^+ - $\cdot CH_2Cl$), 183 (14, M^+ - $\cdot CH_2Cl$ - CH_2=C=O), 120 (75), 106 (15, $C_6H_5NHCH_2^+$), 43 (100,CH_3CO^+).

5.3.15 *N*-[4-[*N'*,*N'*-Bis(2-chloroethyl)amino]phenyl]trifluoroacetamide (12)

N,*N*-Bis(2-chloroethyl)-4-amino-aniline (1.25 g, 4.6 mmol) was suspended in DCM (25 cm^3) and shaken with triethylamine (1.6 cm^3, 11.5 mmol). Trifluoroacetic anhydride (3.2 cm^3, 23 mmol) was added with vigorous shaking. After 20min. the organic layer was washed with water (25 cm^3) and dried over anhyd. $MgSO_4$. After removing the solvent, the dark residue was recrystallised from ether/n-hexane (1:1, v/v). The product was further purified by silica column chromatography (ether/n-hexane (1:1,v/v)) and further recrystallised from ether/n-hexane (1:1, v/v) to give shining silver crystals. Yield 0.3 g (20%); m.p. 101-103°C (lit.[40] m.p. 109-110°C) (Found: C, 44.1; H,4.1; N,8.4. $C_{12}H_{13}Cl_2F_3N_2O$ requires: C, 43.8; H, 4.0; N, 8.5%); λ_{max} (DMSO) 290nm (logε= 4.22); ν_{max} (KBr disc) 3300 (NH), 1700 (C=O, amide), 1620 (ArH), 1600, 1520 (ArH), 1360, 1150, 820 cm^{-1}; δ^1H ($(CD_3)_2CO$) 3.8 (8H, s, CH_2), 6.8 - 7.6 centred at 7.2 (4H, ABq, J=10.5Hz, Ar*H*), 10.1 ppm (1H, br s, N*H*); m/z (EI) 328 (5%,

M^+), 293 (7, M^+-Cl), 279 (47, M^+ - CH_2Cl), 119 (30), 91 (14, $C_7H_7^+$), 69 (27,CF_3^+), 65 (45), 63 (100).

5.3.16 *N*-[4-[*N'*,*N'*-Bis(2-methanesulphonyloxyethyl)amino]phenyl] acetamide (57)

N,*N*-Bis(2-methanesulphonyloxyethyl)-4-amino-aniline methanesulfonate salt (56) (0.7 g, 1.6 mmol) was suspended in dry pyridine (30 cm^3) and shaken with triethylamine (0.6 cm^3, 4.6 mmol), at room temperature. When all the solid had dissolved, the mixture was cooled to 0 to -5°C. Acetic anhydride (1.2 cm^3, 12.7 mmol) was added with vigorous shaking, and the mixture was stirred for 3h at 0 to -5°C. The pyridine was removed under vacuum and the residue washed with a saturared solution of $NaHCO_3$ (20 cm^3) and extracted with DCM (30 cm^3). The organic extract was further washed with water (20 cm^3) and dried over anhyd. $MgSO_4$. After vacuum evaporation of the solvent, a yellow residue was obtained which was submitted to silica column chromatography using ethyl acetate as eluent. The product was obtained after vacuum evaporation of the solvent as a syrup. It was clearly impure and proved impossible to recristallise. Yield 0.3 g (54 %); m.p. 111°C (decomp.); ν_{max} (thin film) 3300 (NH), 1670 (C=O, amide), 1600 (ArH), 1520 (ArH), 1350 (OSO_2CH_3), 1175 (OSO_2CH_3), 800 (ArH) cm^{-1}; δ^1H ($(CD_3)_2CO$) 2.0 (3H, s, NHC(O)C*H*$_3$), 3.0 (6H, s, SO_2CH_3), 3.8 (4H, t, J=6.3Hz, NC*H*$_2$), 4.4 (4H, t, J=6.3Hz, OC*H*$_2$), 6.8-7.5 centred at 7.15 (4H, ABq, J=10.4Hz, Ar*H*), 9.0 ppm (1H, br s, N*H*); m/z (EI) 394 (1%, M^+), 221 (65, M^+ - ($2xSO_2CH_3$), 163 (28, 4-phenylmorpholine$^{+\cdot}$), 96 (80, $HOS^{+\cdot}$ O_2CH_3), 79 (100, $^+SO_2CH_3$), 43 (28, CH_3CO^+).

5.3.17 *N*-[4-[*N'*,*N'*-Bis(2-acetyloxy)ethyl]amino]phenyl]acetamide (58)

N,*N*-Bis(2-hydroxyethyl)-4-amino-aniline sulphate salt (0.5 g, 1.6 mmol) was suspended in pyridine (35 cm^3), and shaken with triethylamine (0.6 cm^3, 4.8 mmol), at room temperature, for 10min. When most of the solid had dissolved, acetic anhydride (3 cm^3, 32 mmol) was added and the mixture shaken vigorously at room temperature for 24h. The pyridine was removed under vacuum. To the residue, water (15 cm^3) was added and the resultant solution was extracted with DCM (30 cm^3). The organic layer was dried over anhyd. $MgSO_4$, the solvent removed under vacuum and the residue submitted to silica column chromatography using ethyl acetate as eluent. The product was then twice recrystallised from DCM/n-hexane (1:1,v/v) to give shining white crystals. Yield 0.16 g (31%); m.p. 64-66°C (Found: C, 59.3; H, 6.8; N, 8.6. $C_{16}H_{22}N_2O_5$ requires C, 59.6; H 6.9; N, 8.7%); ν_{max} (thin film) 3300 (NH),

2980 (ArH, $COCH_3$), 1745 (C=O, ester), 1670 (C=O, amide), 1610, 1520, 1380 (CH_3, ester), 1230, 1050, 810 (ArH); δ^1H ($(CD_3)_2CO$) 1.9 (6H, s, CH_2COCH_3), 2.0 (3H, s, $NHCOCH_3$), 3.6 (4H, t, J=6.7Hz, CH_2), 4.2 (4H, t, J=6.7Hz, CH_2), 6.8 - 7.5 (4H, ABq, J=10.2Hz, ArH), 8.9 ppm (1H, br s, NH); m/z (EI) 322 (5%, M^+), 249 (M^+-CH_3COCH_2·), 109 (30, $H_2NC_6H_4NH_3^+$), 87 (100, $^+CH_2CH_2COOCH_3$), 43 (50, CH_3CO^+).

5.3.18 *N*-[4-[*N'*,*N'*-Bis(2-hydroxyethyl)amino]phenyl]acetamide (59)

N-[4-[*N'*,*N'*-Bis(2-hydroxy)ethyl]amino]phenyl]acetamide, was obtained by hydrolysis of *N*-[4-*N'*,*N'*-[bis(2-acetyloxy)ethyl]amino]phenyl]acetamide **(58)** (0.3 g, 0.93 mmol) in aqueous 0.01M NaOH (100 cm^3) at 40°C, and the reaction followed by HPLC. After 5h, when reaction was complete, the solution was neutralized with CO_2 to pH 7 and extracted with DCM (150 cm^3). After separation and drying ($MgSO_4$), the solvent was removed under vacuum to give a grey residue. Yield 0.19 g (87%). This product resisted further purification. M.p. 142-150°C (decomp.) (lit.[160] m.p. 141-142.5°C); ν_{max} (KBr disc) 3500 (br, OH), 2950 (NH), 1560 (C=O), 1420, 1060, 840, 640 cm^{-1}; δ^1H (CD_3OD) 2.1 (3H, s, $NHCOCH_3$), 3.5 and 3.6 (8H, dd, J=5Hz, CH_2), 4.9 (3H, s, OH and NH), 6.7-7.4 centred at 7.05 ppm (4H, ABq, J=10Hz, ArH); m/z (EI) 238 (30%, M^+), 207 (100, M^+ - CH_2OH), 163 (31), 119 (20, 163), 93 (13), 43 (30, CH_3CO^+).

5.3.19 *N*-[4-(4-morpholino)phenyl]acetamide (60)

To 4-morpholinoaniline (0.8 g, 4.4 mmol) in freshly distilled THF (20 cm^3), triethylamine (20 cm^3, 143.5 mmol) was added. After cooling to 0 to -5°C, acetyl chloride (0.5 cm^3, 6.16 mmol) was added dropwise. After 1h stirring at room temperature, silica tlc using DCM eluent, showed no presence of starting material. The solvent was lyophylised to give a wet residue, which was dissolved in water (20 cm^3) then extracted with DCM (40 cm^3). The organic extract after separation and drying over anhyd. $MgSO_4$, was evaporated under reduced pressure to give a brown red residue which went brown metallic on standing. The residue was purified by silica column chromatography using DCM as eluent. Yield 0.76 g (78%); m.p. 198-200°C (Found: C, 64.1; H, 7.2; N, 11.6. $C_{12}H_{16}N_2O_2$ requires C, 65.4; H, 7.3; N, 12.7%); ν_{max} (KBr disc) 3300 (NH), 1660 (C=O, amide), 1520, 1380, 1220, 920, 820, cm^{-1}; δ^1H ($CDCl_3$) 2.14 (3H, s, CH_3), 3.11 (4H, t, J=1.4Hz, CH_2), 3.86 (4H, t, J=1.4Hz, CH_2), 6.88-7.38 (4H, ABq, J=3Hz, ArH), 7.41 ppm (1H, br s, NH); m/z (FAB^+) 221 (28, MH^+); m/z (FAB^-) 219 (27, M- H^+); m/z (EI) 220 (90, M^+), 189 (2, M^+-CH_2OH), 177 (29, M^+ - CH_3CO), 162 (32), 120 (100, $NH_2C_6H_4NCH_2$), 43 (29, CH_3CO^+).

5.3.20 *N*-[4-[*N'*,*N'*-Bis(2-hydroxyethyl)amino]phenyl]trifluoroacetamide (65)

N-[4-[*N'*,*N'*-Bis(2-hydroxyethyl)amino]phenyl]trifluoroacetamide was obtained by either acid or base hydrolysis of *N*-[4-[*N'*,*N'*-bis(2-chloroethyl)amino]phenyl]trifluoro acetamide (12). Thus (12) (0.15 g, 0.46 mmol) was dissolved in aqueous DMSO (7:3, v/v) [0.6 cm^3 DMSO + 13.8 cm^3 H_2O + 0.2 cm^3 HCl (1M)] at pH 2. The mixture was stirred overnight at 37°C. When HPLC showed complete disappearance of the starting material, the mixture was neutralized with 1M NaOH (500 µl) and freeze dried. The residue was extracted into ethyl acetate (35 cm^3), washed with water (20 cm^3), dried ($MgSO_4$) and the solvent evaporated under reduced pressure. HPLC of this residue gave a single peak (Rf=17.75min). Yield 0.12g (89%); ν_{max} (KBr disc) 3500 (br, OH), 3300 (NH), 1700 (C=O, amide), 1630, 1600, 1520, 1350, 1170, 820 cm^{-1}; δ^1H ($(CD_3)_2CO$) 2.55 (>2H, br s, O*H*), 3.62 (4H, t, J=1.3Hz, CH_2), 3.75 (4H, t, J=1.3Hz, CH_2), 6.98 - 7.59 (4H, ABq, J=2Hz, Ar*H*), 10.5 ppm (1H, br s, N*H*); m/z (FAB$^+$) 293 (100%, MH^+), 275 (10, MH^+- H_2O), 261 (18, MH^+-CH_3OH), 243 (10), 231(14, MH^+- CH_2=CH_2), 217 (30), 196 (3, MH^+-CF_3CO), 186 (14); m/z (FAB$^-$) 291 (100%, M-H^+), 273 (4, M-H^+-H_2O), 259 (2), 247 (7, 291-CO_2), 230 (5), 215 (7).

5.3.21 *N,N*-Bis(2-hydroxyethyl)-4-amino-aniline (66)

The *N,N*-Bis(2-hydroxyethyl)-4-amino-aniline sulphate salt (2.0 g, 6.4 mmol) after recrystallisation from ethyl alcohol, was dissolved in ethyl alcohol (5 cm^3) and shaken with one equivalent of aqueous NaOH (1M, 6.4 cm^3) for 10min. The reaction was followed by HPLC. On completion, the solution was freeze dried and extracted with ethyl acetate (15 cm^3). The organic solution was washed with water (10 cm^3), dried over anhyd. $MgSO_4$ and the solvent evaporated under vacuum. The residue was impure and proved impossible to purify. Yield 0.43 g (34%); m.p. 85-95°C (lit.[157] m.p. 87-88°C); ν_{max} (KBr disc) 3400 (OH), 3000 (NH), 1620, 1500, 1100 (OH), 620.cm^{-1}; δ^1H ($(CD_3)_2SO$) 3.2 (8H, d, CH_2), 4.9 (1H, br s, N*H* or O*H*), 6.8-7.5 ppm (4H, ABq, J=2Hz, Ar*H*); m/z (FAB$^+$) 197 (14%, MH^+).

5.3.22 4-(*N*-Benzyloxycarbonylamino)benzyl alcohol (85)

4-Aminobenzyl alcohol (80) (1.3 g, 1.1 mmol) was dissolved in ether (100 cm^3) at room temperature. To this solution, water (35 cm^3) and magnesium oxide (3.2 g, 79 mmol) was added. The mixture was cooled to 0°C, and benzyl chloroformate (1.6 cm^3, 11.2 mmol) in ether

(15 cm^3) was added dropwise and the reaction allowed to proceed at 0°C for 1h, and for a further 3h at room temperature. The reaction was followed by silica tlc using ether as eluent (Rf= 0.69). The organic layer was separated, washed with water (30 cm^3), dried over anhyd. $MgSO_4$, and the ether removed using a rotary evaporater. The residue (2.3 g; 85%) was purified by silica column chromatography using ether, and further recrystallised from ether/n-hexane (5:1, v/v) to give a white solid. Yield 1.7 g (64%); m.p. 94-95°C; ν_{max} (KBr disc) 3300 (OH and RCONHR'), 1710 (C=O, carbamate), 1600, 1540, 1420, 1310, 1240, 1080, 1010, 840, 780, 740 and 690 cm^{-1}; δ^1H ($CDCl_3$) 2.1 (1H, br s, O*H*), 4.5 (2H, s, ArCH_2OH), 5.2 (2H, s, ArCH_2OCO), 6.9 (1H, br s, N*H*), 7.3 (8H, m, Ar*H*); m/z (EI) 257 (48 %, $M^{+\cdot}$), 213 (42, $M^{+\cdot}$ - CO_2), 149 (43, $M^{+\cdot}$ - $PhCH_2OH$), 108 (56, $PhCH_2OH^{+\cdot}$), 107 (60, $PhCH{=}OH^+$), 91 (100, $C_7H_7^+$), 79 (66), 77 (53), 65 (13), 51 (26).

5.3.23 4'-(*N*'-Benzyloxycarbonylamino)benzyl *N*-(4-nitrophenyl) carbamate (75)

Compound (85) (0.63 g, 2.4 mmol) was dissolved in dry ACN (80 cm^3) and dry DCM (20 cm^3). Then, 4-nitrophenyl isocyanate (0.49 g, 2.6 mmol) was added, and the reaction allowed to proceed for 12h at room temperature. Subsequently, the temperature was increased to 40°C and the reaction allowed to continue for a further 3h. The reaction was monitored by silica tlc, using ether/n-hexane (4:1, v/v) as eluent (Rf=0.6). The solvents were vacuum evaporated, the residue was added to water (15 cm^3) and then extracted with ether (45 cm^3). The ether extract was dried over anhyd. $MgSO_4$ and the solvent vacuum evaporated. The impure residue (0.71 g, 69%) was purified by silica column chromatography using ether as eluent, and further recrystallised from pet. ether (40-60°C). Yield 0.21 g (20%); m.p. 159-160°C (Found: C, 62.0; H, 4,6; N, 9.6. $C_{22}H_{19}N_3O_6$ requires: C, 62.7; H, 4.5; N, 9.9%); ν_{max} (KBr disc) 2950, 2260, 1730 (C=O, carbamate), 1670, 1600, 1510, 1350, 850, 745 and 680 cm^{-1}; δ^1H ($CDCl_3$) 5.25 and 5.30 (4H, ds, ArCH_2), 7.30 (9H, m, Ar*H*), 7.85 (4H, ABq, J=10.3Hz, Ar*H*); m/z (FAB^-) 420 (37%, $M\text{-}H^+$).

5.3.24 4'-(*N*'-Benzyloxycarbonylamino)benzyl *N*-(4-trifluoromethylphenyl) carbamate (76)

The protected amino alcohol (85) (0.3 g, 1.17 mmol) was dissolved in dry ACN (50 cm^3) and dry DCM (30 cm^3). To this solution, 4-trifluoromethylphenylisocyanate (183 µl, 0.28 mmol) was added, and the reaction allowed to proceed at room temperature for 2h, then was warmed to 40°C for 16h. The reaction was monitored by silica tlc using ether/n-hexane (2:1,v/v) as

eluent (Rf=0.6). On completion, the solvent was evaporated under reduced pressure to give a residue which was purified by silica column chromatography using ether/n-hexane (2:1,v/v) as eluent. The pure fraction after evaporation of the solvent gave a solid white residue (0.22 g, 43%); m.p. 167-170.5°C; ν_{max} (KBr disc) 3350, 1720 (C=O, carbamate), 1610, 1540, 1420, 1340, 1420, 1340, 1240, 1170, 1120, 1080, 840, 740 cm^{-1}; δ^1H ($CDCl_3$ + $(CD_3)_2CO$)) 5.1 and 5.15 (4H, ds, CH_2), 7.2-7.7 (13H, m, Ar*H*), 8.6 (1H, br s, N*H*), 8.9 (1H, br s, N*H*); m/z (EI) 444 (0.1%, M^+), 400 (0.3, M^+-CO_2), 336 (0.1, M^+- $PhCH_2OH$), 257 (4, M^+ for (85)), 187 (28, M^+ for $O=C=NC_6H_4CF_3$), 132 (54), 91 (100, $C_7H_7^+$).

5.3.25 4-(*N*-Triphenylmethylamino)benzyl alcohol (91)

Following a general procedure,[192] 4-aminobenzyl alcohol (80) (0.8 g, 6.5 mmol), was dissolved in dry DCM (120 cm^3), and triethylamine (0.9 cm^3, 6.5 mmol) plus triphenylmethyl chloride (1.81 g, 6.5 mmol) was added. The mixture was stirred at 35°C, under N_2, for 4h, and then washed with water (35 cm^3), dried over anhyd. $MgSO_4$, and evaporated at reduced pressure, to give an impure residue as a foam (2.36 g, 99.5%). The reaction was followed by silica tlc using ether/n-hexane (2:1, v/v) as eluent (Rf=0.38). The residue was purified by silica column chromatography using ether/n-hexane (2:1,v/v) as eluent, followed by recrystallisation from DCM/n-hexane (3:1, v/v), to give a white solid. Yield 1.8 g (77%); m.p. 188-190°C (Found: C, 83.9; H, 6.3; N, 3.7. $C_{26}H_{23}NO$ requires: C, 85.5; H, 6.3; N, 3.8%); ν_{max} (KBr disc) 3620 (OH), 3440 (NH), 1620, 1500, 1450, 1330 (COH), 1010, 1040 (COH), 840, 760, 710 cm^{-1}; δ^1H ($(CD_3)_2SO$) 3.4 (1H, s, N*H*), 4.2 (2H, d, CH_2), 4.75 (1H, t, O*H*), 6.55 (4H, ABq, J=8.7Hz, Ar*H*), 7.25 (15H, m, *Ph*); m/z (EI) 365 (2%, $M^{+\cdot}$), 347 (0.2, M^+-H_2O), 334 (0.3, M^+-CH_2OH), 288 (2, $M^{+\cdot}$ - $C_6H_5\cdot$, $Ph_2CN^+HPhCH_2OH\cdot$), 243 (100, Ph_3C^+), 165 (48, PhC_7H_6).

5.3.26 4'-(*N*'-Triphenylmethylamino)benzyl *N*-(4-nitrophenyl) carbamate (77)

The protected alcohol (91) (0.46 g, 1.26 mmol) was dissolved in dry THF (60 cm^3). The mixture was cooled to 0°C and n-butylithium (0.8 cm^3, 1.3 mmol, 1.1 eq, 1.6M solution in hexane) was added dropwise and the reaction allowed to proceed for 15min under nitrogen. Then, 4-nitrophenylisocyanate was added at 0°C in four portions (0.23 g, 1.39 mmol, 1.1 eq) at intervals of 2h, after which the reaction was allowed to proceed at room temperature, under nitrogen, for a further 1h. The reaction was followed by silica tlc, using ether/n-hexane (2:1, v/v) as eluent (Rf (77) =0.61, Rf (91) =0.37). On completion, the solvent was evaporated under

reduced pressure, and the residue extracted into DCM (60 cm^3). The DCM solution was washed with sat. aqueous ammonium chloride (pH 7; 30 cm^3), water (30 cm^3), dried over anhyd. $MgSO_4$, and the solvent evaporated under reduced pressure. The residue was purified by a silica flash chromatography using ether as eluent, followed by a silica column chromatography using ether/n-hexane (2:1, v/v) as eluent. The fractions containing the product were combined and the solvent evaporated under reduced pressure to give a yellow residue. Yield 0.5 g (76%); m.p. 79-88°C (Found: C, 76.7; H, 5.7; N, 6.9. $C_{33}H_{27}N_3O_4$ requires: C, 74.8; H, 5.1; N, 7.9%); ν_{max} (KBr disc) 3400 (NH), 1730 (C=O, carbamate), 1600, 1500, cm^{-1}; δ^1H ($CDCl_3$) 4.4 (2H, s, CH_2), 5.1 (1H, s, N*H*), 6.35-6.9 (4H, ABq, J=2.0Hz, Ar*H*), 6.6-8.1 (4H, ABq, J=2.2Hz, Ar*H*), 7.3 (17H, m, Ar*H*); m/z (FAB$^+$) 485 (0.1%, M^+-CO_2), 243 (100, Ph_3C^+); m/z (EI) 485 (0.1%, M^+-CO_2), 364 (10, Ph_3C^+·NHPhCHOH), 347 (52, Ph_3CN^+·$PhCH_2$), 334 (8, $Ph_3CNC_6H_5^+$), 243 (100, Ph_3C^+), 165 (65).

5.3.27 4-(*N-tert*-Butoxycarbonylamino)benzyl alcohol (93)

Following a literature procedure,[174] a mixture of 4-aminobenzyl alcohol (0.41 g, 3.3 mmol) and di-t-butyl dicarbonate (896 µl, 3.9 mmol), in dry THF (25 cm^3), was stirred at 25°C, for 17h. The reaction was monitorated by silica tlc using ether as eluent (Rf(80)=0.44, Rf(93)=0.81). The solvent was evaporated under reduced pressure and the yellow-brown oily residue dissolved in ether (10 cm^3), washed with 5% aqu. sodium bicarbonate (2 x 10 cm^3) and dried over anhyd. $MgSO_4$. The yellow-brown solid residue after vacuum removal of the solvent was chromatographed on a silica column with elution by ether. The fractions containing the product were combined, the solvent evaporated under reduced pressure and the residue vacuum dried. Yield 0.55 g (75%); m.p. 79-81°C (Found: C, 64.59; H, 7.72; N, 6.22. $C_{12}H_{17}NO_3$ requires: C, 66.55; H, 7.67; N, 6.27%); ν_{max} (KBr disc) 3480 (OH), 3380 (NH), 1700 (C=O, carbamate), 1610, 1530, 1420, 1370, 1320, 1240, 1160, 1000, 830 and 770 cm^{-1}; δ^1H ($CDCl_3$) 1.48 (9H, s, $C(CH_3)_3$), 3.77 (1H, s, O*H* or N*H*), 4.47 (2H, s, CH_2), 7.12-7.27 (4H, m, ABq, J=2.2 Hz, Ar*H*), 7.29 (1H, s, N*H* or O*H*); m/z (EI) 223 (18%, M^+), 167 (75, M^+ - $C(CH_3)_3^+$), 138 (29), 132 (21), 123 (35, $^+$·NH_2PhCH_2OH), 122 (33, H_2N^+·PhCHOH), 106 (16, $H_2N^+PhCH_2$), 94 (18, $C_6H_6O^+$·), 77 (16), 57 (100, $C(CH_3)_3^+$), 41 (37).

5.3.28 4'-(*N'-tert*-Butoxycarbonylamino)benzyl *N*-(4-nitro-phenyl) carbamate (78)

Compound (93) (0.7 g, 3.1 mmol) in dry THF (80 cm^3) was cooled in an ice bath and n-butyllithium (2.15 cm^3, 3.4 mmol), was added dropwise. After 15 min, 4-nitrophenyl-

isocyanate (0.56 g, 3.4 mmol), dissolved in THF (5 cm^3), was added in 3 fractions, with the temperature mantained at 0°C. After these additions, the mixture was stirred for 14h at room temperature. When silica tlc using ether/n-hexane (2:1, v/v) as eluent (Rf(93)=0.34, Rf(78)=0.63) indicated complete disappearance of the starting material, the solvent was vacuum evaporated to give a yellow residue. This residue was taken up in ether (30 cm^3), washed with satd. aqu. ammonium chloride (15 cm^3), then water (15 cm^3). After separation, the organic solvent was evaporated under vacuum to give a residue, which was dissolved in ether and purified by silica column chromatography using ether/n-hexane (2:1, v/v) as eluent. The fractions containing the compound were combined, the solvent vacuum evaporated, and the resultant yellow solid residue recrystallised twice from ether/n-hexane (1:1, v/v). Yield 0.77 g (64%); m.p. 158-161°C (Found: C, 58.5; H,5.4; N, 10.6. $C_{19}H_{21}N_3O_6$ requires: C, 58.9; H, 3.5; N, 10.8 %); λ_{max} (DMSO) 242, 319; ν_{max} (KBr disc) 1760 and 1715 (C=O, carbamate), 1620, 1540, 1360, 1230 cm^{-1}; δ^1H ($CDCl_3$) 1.52 (9H, s, $C(CH_3)_3$), 5.15 (2H, s, CH_2), 6.55 (1H, s, NH), 7.17 (1H, s, NH), 7.32-7.38 and 7.32-8.19 ppm (8H, 2 ABq, J=2.0 Hz, ArH); m/z (EI) 387 (0.2%, $M^{+}\cdot$), 343 (0.4, M^{+}-CO_2), 331 (0.4, M^{+}-$C(CH_3)_3$), 224 (2, $(CH_3)_3OCONHC_6H_4CH_2OH_2^{+}$), 182 (2, $C(O)OHNHC_6H_4NO_2$), 167 (8, $COOHNHC_6H_4CH_2OH$), 150 (27), 138 (36, $H_2NC_6H_4NO_2$), 132 (20), 122 (6, $C_6H_5NO_2$), 106 (34), 92 (19), 65 (46), 57 (100, $(CH_3)_3C^{+}$), 44 (50, CO_2).

5.3.29 4-(*N*-Acetylamino)benzyl methyl ester (95)

4-Aminobenzyl alcohol (0.52 g, 4.1 mmol) in dry pyridine (15 cm^3) was reacted with acetic anhydride (3 cm^3, 32 mmol) at room temperature, with vigorous stiring, for 12h, and for a further 1h, at 40°C. The reaction was followed by silica tlc, using ether as eluent (Rf=0.2), and when all the starting material had disappeared, the pyridine was removed at reduced pressure and low temperature. The residue was dissolved in DCM (35 cm^3) washed with water (15 cm^3) and then dried over anhyd. $MgSO_4$. After removing the solvent under reduced pressure, a white solid was obtained. Yield 0.79 g (93%); m.p. 101-103°C (lit.[193] m.p. 102°C) (Found: C, 63.5; H, 6.3; N, 6.7. $C_{11}H_{13}NO_3$ requires: C, 63.7; H, 6.3; N, 6.7%); ν_{max} (KBr disc) 3300, 1760 (C=O, ester), 1680 (C=O, amide), 1620, 1560, 1420, 1380, 1330, 1250, 1020, 980, 840, 980 cm^{-1}; δ^1H ($(CD_3)_2CO$) 2.02 (3H, s, $OCOCH_3$), 2.07 (3H, s, $NHCOCH_3$), 5.01 (2H, s, $PhCH_2OCO$), 7.51 (4H, ABq, J=11.0Hz, ArH), 9.21 (1H, br s, NH); m/z (EI) 207 (18%, $M^{+}\cdot$), 165 (16, M^{+}-CH_2=C=O, $H_2N^{+}\cdot C_6H_4CH_2OC(O)CH_3$), 123 (42, $^{+}\cdot NH_2PhCH_2OH$), 106 (100, $H_2N^{+}PhCH_2$), 94 (34, $C_5H_4CHO^{+}\cdot H$), 77 (31), 65 (10), 57 (9), 43 (35, CH_3CO^{+}).

5.3.30 4-(*N*-Acetylamino)benzyl alcohol (96)

4-*N*-(Acetylamino)benzyl methyl ester **(95)** (0.69 g, 3.3 mmol), was dissolved in ethanol (7 cm^3), water (20 cm^3), and NaOH (1M, 4.5 cm^3). The mixture was stirred at 40°C, for 6h, to effect disappearance of the starting material (followed by HPLC). The solution was then neutralized to pH 7 with CO_2, concentrated to 5 cm^3, and extracted into ethyl acetate (35 cm^3). The organic extract was washed with satd. aqueous sodium bicarbonate (25 cm^3), water (15 cm^3), and dried over anhyd. $MgSO_4$. The solvent was then evaporated under reduced pressure to give a white solid. Yield 0.42 g (76%); m.p. 115-117.5°C (lit.[194] m.p. 120-1°C); ν_{max} (KBr disc) 3450 (OH), 1680 (C=O, amide), 1620, 1560, 1520, 1420, 1380, 1330, 1280, 1010, 840, 800, 580 cm^{-1}; δ^1H ($(CD_3)_2CO$) 2.1 (3H, s, $COCH_3$), 4.2 (1H, t, J=7.0Hz, O*H*), 4.6 (2H, d, J=7Hz, $ArCH_2OH$), 7.2-7.7 (4H, ABq, J=11.0Hz, Ar*H*), 9.1 (1H, br s, N*H*); m/z (EI) 165 (77%, $M^{+\cdot}$), 123 (100, $M^{+\cdot}$- CH_2=C=O), 106 (99, $^+NH_2PhCH_2$), 94 (76, $C_6H_6O^{+\cdot}$), 77 (38, $C_6H_5^+$), 43 (95, CH_3CO^+).

5.3.31 4'-(*N*'-Acetylamino)benzyl *N*-(4-nitrophenyl) carbamate (26)

4-(*N*-Acetylamino)benzyl alcohol **(96)** (0.24 g, 1.5 mmol) was dissolved in ACN (15 cm^3) and DCM (10 cm^3), and 4-nitrophenylisocyanate (0.3 g, 1.8 mmol) was added. The reaction was allowed to proceed for 4h at room temperature, and another 5h, at 40°C. Loss of the starting material was followed by silica tlc, using ethyl acetate as eluent (Rf **(26)** =0.81, Rf **(96)**=0.28). The solvents were removed under vacuum to give a yellow solid, which was purified by silica column chromatography using DCM as eluent. Yield 0.34 g (69%); m.p. 200-201°C (Found: C, 57.3; H, 4.4; N, 13.2. $C_{16}H_{15}N_3O_5$ requires: C, 58.4; H, 4.6; N, 12.8%); ν_{max} (KBr disc) 3344 (NH), 1722 (C=O, carbamate), 1700 (C=O, amide), 1600, 1534, 1412, 1222, 1052, 850 cm^{-1}; δ^1H (EtOD+ $(CD_3)_2SO$) 2.09 (3H, s, $COCH_3$), 4.40 (2H, s, N*H*), 5.16 (2H, s, CH_2OCO), 7.32-8.24 (4H, ABq, J=9.0 Hz, Ar*H*), 7.61-8.24 ppm (4H, ABq, J=9.0 Hz, Ar*H*); m/z (FAB^-) 328 (79%, $M\text{-}H^+$), 259 (13), 213 (28), 137 (60, $NHPhNO_2^-$), 103 (28), 91 (18).

5.3.32 4'-Aminobenzyl *N*-(4-nitrophenyl) carbamate, picrate salt (94)

To compound **(78)** (0.3 g, 0.79 mmol) in THF (6 cm^3) at room temperature, was added dropwise 2-mercaptoethane sulphonic acid in glacial acetic acid (4 cm^3, 20% w/v, 5.6 mmol) [Prepared by passing the sodium salt of 2-mercaptoethanesulphonic acid (0.64 g, 3.8 mol) dissolved in water (5 cm^3) through a Dowex $50H^+$ ion exchange resin followed by lyophilisation of the

solution to give the dry acid (0.54 g, 3.8 mol), which was then dissolved in glacial acetic acid (2.7 cm^3)]. After 1h with stirring at room temperature, silica tlc using ether eluent showed complete disappearance of the starting material. An aqueous solution of picric acid (20 cm^3, 1%,w/w, 0.9 mmol) was then added and the solution cooled to 0-5°C. After a few min, an orange precipitate formed and the solution was left at 0-5°C overnight. The precipitate was filtered, washed with ice-water (0-5°C) and dried. Yield 0.34 g (84%); m.p. 120-122°C (Found: C, 46.5; H, 3.2; N, 15.8. $C_{20}H_{16}N_6O_{11}$ requires: C, 46.5; H, 3.1; N, 16.3%); ν_{max} (KBr disc) 1760 (C=O, carbamate), 1640, 1580, 1520, 1350, 1240, 1080 cm^{-1}; δ^1H $(CD_3)_2SO$) 5.26 (2H, s, ArCH_2OCO), 6.62 - 7.45 (4H, ABq, J=2Hz, Ar*H*), 7.43 (>2H, m, N*H*), 7.71 - 8.22 (4H, ABq, J=2Hz, Ar*H*), 8.63 ppm (2H, s, Ar*H*), 10.08 (1H, br s, N*H*); m/z (FAB^+) 289 (1%, $NO_2C_6H_4NHCOOCH_2C_6H_4NH_3^+$), 123 (39, $^+NH_2PhCH_2OH$), 105 (100); m/z (FAB^-) 243 (40), 228 (62, $(NO_2)\ 3C_6H_2O^-$), 212 (22), 199 (49), 121 (60), 77 (100), 59 (10, CH_3COO^-).

5.3.33 4'-Aminobenzyl *N*-(4-nitrophenyl) carbamate (74)

The picrate (94) (0.13 g, 0.25 mmol) in aqu. ethanolamine (15 cm^3, 10%, w/w), was stirred for 10min at room temperature, and then extracted into ether (45 cm^3). Completion of the reaction was monitored by silica tlc using ether/n-hexane (2:1, v/v) (Rf(74)=0.15). The ether extract was washed with water (30 cm^3) and dried over anhyd. $MgSO_4$. The solvent was vacuum evaporated and the residue purified by silica column chromatography using ether as eluent. The fractions containing the compound were combined, the solvent vacuum evaporated to give a solid yellow residue. Yield 0.04 g (55%); m.p. 110-140°C (decomp.); ν_{max} (KBr disc) 3400 (NH), 1760 (C=O, carbamate), 1630, 1610, 1520, 1340, 1230 cm^{-1}; δ^1H ($CDCl_3$ + $(CD_3)_2SO$) 3.26 (>2H, s, N*H*), 4.47 (1H, s, N*H*), 5.04 (2H, s, CH_2), 6.65 - 7.17 (4H, ABq, J=2.1 Hz, Ar*H*), 7.70-8.11 (4H, ABq, J=2.3 Hz, Ar*H*), 10.10 (1H, br s, N*H*); m/z (FAB^-) 286 (11%, $M\text{-}H^+$), 259 (12), 225 (5, M^+- (NH_2+NO_2)), 213 (10, $^+NHPhCH_2OH$), 197 (12, M^+- (CO_2+NO_2)), 181 (15), 167 (18), 155 (19), 137 (38, $NHPhNO_2^-$), 91 (8), 46 (3); (FAB^+) 273 (7), 200 (6), 145 (10).

5.3.34 4-Nitrobenzyl *N*-phenyl carbamate (104)

Phenylisocyanate (0.78 cm^3, 7.1 mmol) in ACN (5 cm^3) added dropwise to 4-nitrobenzyl alcohol (105) (0.91 g, 5.9 mmol) in ACN (20 cm^3), was allowed to react at room temperature, for 48h. The reaction was followed by silica tlc using ether/n-hexane (1:1, v/v) as eluent (Rf= 0.54). The solvent was vacuum evaporated and the residue after washing with water

(15 cm^3) was extracted into DCM (35 cm^3). The product was purified by silica flash chromatography using ether/n-hexane (1:1,v/v) as eluent, followed by silica column chromatography using the same eluent. Yield 1.14 g (71%); m.p. 146-148°C; (Found: C, 62.1; H, 4.5; N, 10.2. $C_{14}H_{12}N_2O_4$ requires: C, 61.8; H, 4.5; N, 10.3%); ν_{max} (KBr disc) 3300 (NH), 1740 (C=O, carbamate), 1640, 1560, 1460, 1380, 1360, 1320, 1280, 1240, 740, 700 cm^{-1}; δ ^1H ($CDCl_3$) 5.2 (2H, s, CH_2), 6.8 (1H, br s, N*H*), 7.3 (5H, m, Ar*H*), 7.5-8.2 (4H, ABq, J=8.8Hz, Ar*H*); m/z (EI) 272 (60%, M^+), 255 (8, M^+ - OH), 228 (15, M^+ - CO_2), 153 (13, 4-nitrobenzyl alcohol$^{+\cdot}$), 136 (100, $ONC_6H_4CHO^{+\cdot}$·H - OH), 119 (90, phenylisocyanate$^{+\cdot}$), 106 (84, H_2N^+=C_6H_4=CH_2), 89 (59), 78 (74), 64 (36), 51 (37), 30 (45, NO^+ or CH_2=$O^{+\cdot}$).

5.3.35 Benzyl *N*-(4-methylphenyl) carbamate (112)

4-Toluidine (0.71 g, 6.6 mmol) in ether (100 cm^3) and water (50 cm^3) was mixed with MgO (2.5 g, 62.0 mmol). The solution was cooled to 0°C, and benzylchloroformate (1.1 cm^3, 7.9 mmol) added dropwise, with vigorous stirring. After 90min, silica tlc using ether/ n-hexane (2:1, v/v) show complete disappearance of the starting material. The organic layer was separated, washed with water (35 cm^3), dried over anhyd. $MgSO_4$, and the solvent vacuum evaporated. The residue was purified by silica column chromatography using DCM as eluent. Yield 1.4 g (89%); m.p. 78-79°C; ν_{max} (KBr disc) 3316, 1725 (C=O, carbamate), 1620, 1560, 1250, 1080 cm^{-1}; δ^1H ($CDCl_3$) 2.2 (3H, s, CH_3), 5.1 (2H, s, CH_2), 6.8-7.4 (4H, ABq, J=9.2 Hz, Ar*H*), 7.3 ppm (5H, m, *Ph*); m/z (FAB^+) 242 (2%, MH^+), 108 (15); m/z (FAB^-) 240 (5%, $M\text{-}H^+$).

5.3.36 Ethyl *N*-(4-nitrophenyl) carbamate (113)

4-Nitrophenylisocyanate (0.5 g, 3 mmol) and ethanol (100 cm^3) were stirred at 40°C for 1h. The reaction was followed by silica tlc using DCM as eluent. The excess alcohol was evaporated under vacuum to give a solid residue. Yield 0.6 g, (98%). The residue was purified by flash chromatography using DCM. Yield 0.41 g, (67%); .m.p. 134-135°C (Found: C, 51.6; H, 4.8; N, 13.2. $C_9H_{10}N_2O_4$ requires: C, 51.4; H, 4.8; N, 13.3%); ν_{max} (KBr disc) 3394, 1743 (C=O, carbamate), 1605, 1540, 1500, 1330, 1210, 1060 cm^{-1}; δ^1H ($CDCl_3$) 1.3 (3H, t, CH_3), 4.2 (2H, q, CH_2), 7.3 (1H, br s, N*H*), 7.5-8.3 ppm (4 H, ABq, J=9.0Hz, Ar*H*); m/z (EI) 210 (87%, M^+), 165 (11, M^+ - NO_2 or C_2H_5OH), 151 (63), 138 (38), 108 (68), 105 (25), 92 (30), 29 (100, $C_2H_5^+$).

5.4 Reaction methods

5.4.1 Kinetics of mustard (5) and (38) - (41) hydrolysis followed by autotitration

This method was used to follow the hydrolysis of simple mustards **(5)**, **(38)**, **(39)**, **(40)**, **(41)**.

Usually, a solution of the substrate mustard in DMSO (100 μl, $8x10^{-2}$ M) was injected into aqueous DMSO [either 99:1, 9:1, or 7:3 (v/v), depending on the substrate] (25 cm^3) contained in a thermostatted reaction vessel of the auto-titrator at 37°C. Two electrodes (calomel and glass) were immersed in the solution. The initial pH of the solution was adjusted to pH 7.0 or 9.0 with standardised NaOH (5 mM) which was also used to titrate the acid released. The concentration of the substrate mustard in the reaction solution was *ca.* $3.2x10^{-4}$ M. With the solution vigorously stirred and under a continuous flow of nitrogen, the reaction was followed by autotitration of the acid released by hydrolysis of the mustard with the standardised 5 mM NaOH. The volume of NaOH added was monitored and plotted by the autotitrator as a function of time (***Figure 5.1***). Readings were continued until no further addition was apparent, when completion of the reaction was assumed (t_∞).

Values of k_O {Rate = k_O [substrate]} were calculated from plots of log (v_∞ - v_t) versus time, where v_t and v_∞ = vol. of 5mM NaOH added at time = t and infinity, respectively.

5.4.2 Kinetics of mustard (10) - (12) hydrolysis by UV-visible spectrophotometry

The hydrolysis of substrates **(10)**, **(11)** and **(12)** in aqueous DMSO (99:1, v/v) at pH 2 and 12 and in phosphate buffer/DMSO (1%, v/v) at pH 7.4, all at 37°C were also followed by UV-visible spectrophotometry.

Usually, a solution of the substrate mustard in DMSO (30 μl, $1.2x10^{-2}$M) was injected into an aqueous solution at pH 2, or 12 (adjusted with either HCl (1M) or NaOH (1M)) to give a final aqueous DMSO solution (99:1, v/v), (3 cm^3), contained in a cuvette thermostatted at 37°C. This gave a substrate concentration of ca. $1x10^{-4}$ M.

The UV-visible spectrum of the cell contents was monitored from 200-400 nm at 15 or 30 min timed intervals, depending on the reaction rate.

A similar procedure was followed for reactions in phosphate buffer (0.05M)/DMSO (99:1, v/v; pH 7.4) and in aqueous DMSO (9:1, v/v; pH 2) containing either NaCl (0.5M) or

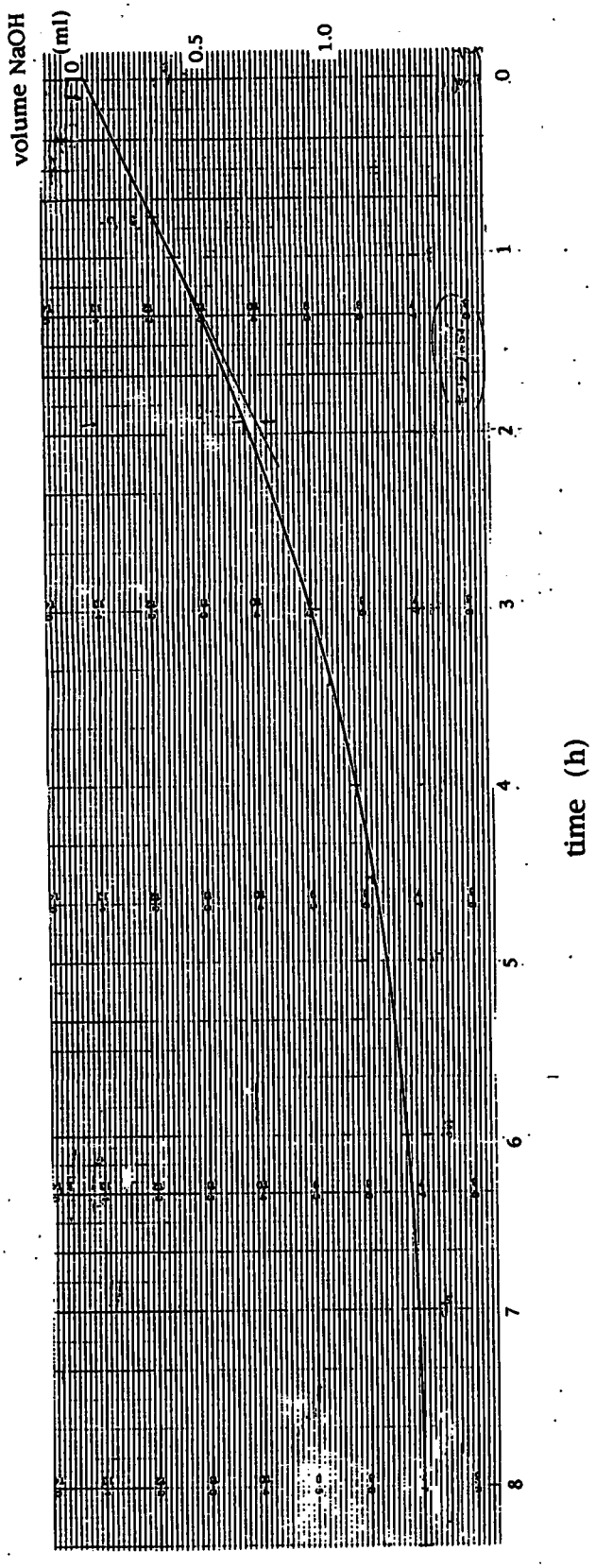


Figure 5.1 *Hydrolysis of mustard (**5**) in aqueous DMSO (7:3, v/v) at pH 9 and 37°C followed by autotitration of HCl released; $[5]_{init.}$ ca. $3.2x10^{-4}M$*

$NaClO_4$ (0.5M). For these substrate in DMSO (10 µl, $1.5x10^{-2}$M) was injected into the solution given a final concentration of $5x10^{-5}$M.

Reactions were followed for 10 half-lives and k_o (rate = k_o [substrate]) was calculated from reaction half-lives ($t_{1/2}$).

Table 5.1 shows the λ_{max} and extinction coefficients of the substrate mustards and the hydrolysis products identified at different pH.

Table 5.1

λ_{max} of mustards and hydrolysis products in aqueous DMSO (1%,v/v) at different pH

Compound	λ_{max} / log ε				
pH:	0	2	7	12	>12
N-[4-[*N'*,*N'*-Bis(2-chloroethyl)amino] phenyl]acetamide **(11)**	246/ 4.09	257	270	270/ 4.11	
[*N*-[4-[*N'*,*N'*-Bis(2-chloroethyl)amino] phenyl]trifluoroacetamide **(12)**	246/ 4.05	285/ 4.08	286	279/ 4.00	
N,*N*-Bis(2-chloroethyl)-4-amino-aniline hydrochloride **(54)**	258	258/ 4.18	258	254/ 4.00	254
N-[4-[*N'*,*N'*-Bis(2-hydroxy)ethyl]amino] phenyl]acetamide **(59)**		245/ 3.74	271	271/ 3.79	
4-morpholinophenylacetamide **(60)**		244/ 4.14	258	258/ 4.10	
4-Morpholinoaniline **(61)**		246	246	220	
N-[4-[*N'*,*N'*-Bis(2-hydroxyethyl)amino] phenyl]trifluoroacetamide **(65)**		248, 290			
N,*N*-Bis(2-hydroxyethyl)-4-amino-aniline **(66)**		260/ 3.65	258	253/ 4.08	253
Half-hydrolysed mustard **(67)**		278			
Half-hydrolysed mustard **(68)**		297			
Half-hydrolysed mustard **(69)**		260			

5.4.3 Kinetics of mustard (5), (10) - (12), (40), (41) and (58) hydrolysis and analysis of products followed by HPLC

5.4.3.1 Analysis of hydrolysis products of mustard (40) and kinetics of mustard (41) hydrolysis in aqueous DMSO

N,N-Bis(2-thiocyanatoethyl)aniline **(40)** or *N,N*-bis(2-nitratoethyl)aniline **(41)** in DMSO (100 µl, $8x10^{-2}$M) were injected into aqueous DMSO (7:3, v/v) (25 cm^3) [$3.2x10^{-4}$M]. The solution was adjusted to pH 9.0 with 5mM NaOH using the autotitrator (see Section 5.4.1). The solution was thermostatted at 37°C and kept under vigorous stirring and constant pH 9.0 by the autotitrator.

For **(40)**, an aliquot (10 µl) of the reaction mixture was taken after 72 h and for **(41)**, aliquots (10 µl) of the reaction mixture were taken at regular intervals, over 97 h, and analysed by HPLC against authentic *N,N*-bis(2-hydroxyethyl)aniline **(37)**, 4-phenylmorpholine **(45)** and starting material either **(40)** or **(41)**.

Two different HPLC conditions were used for the assays: Condition A for the hydrolysis products of **(40)** and Condition B for the hydrolysis products and monitoring of **(41)**. The retention times for authentic **(37)**, **(40)**, **(41)** and **(45)** are summarised in ***Table 5.2***, together with the HPLC conditions.

Table 5.2

*HPLC conditions, retention times, and λ_{max} for authentic materials used in the product analysis of mustards **(40)** and **(41)***

Condition A) Column: C_8-APEX II OCTYL 5µ; 25cm x 4.6mm i.d.
Temperature: 25°C
Eluent: Isocratic- aqueous 0.01M KH_2PO_4 / ACN (5: 4.5, v/v)
Flow rate: 1 cm^3/min
Detection: UV 263 nm
Injection: 10 µl

Condition B) Column: C_8-APEX II OCTYL 5µ; 25cm x 4.6mm i.d.
Temperature: 30°C
Eluent: Two component gradient using Solution B1- aqueous.
0.01M KH_2PO_4 / ACN (6:4, v/v) and
Solution B2- aqueous 0.01M KH_2PO_4/ACN (3:7, v/v)
Gradient program: 100% B1 to 100% B2 in 10min
Flow rate : 1 cm^3/min

Detection: UV 263 nm

Injection : 10 µl

Compound	Retention time (min) / HPLC condition		λ_{max} in HPLC eluent
N,N-Bis(2-hydroxyethyl)aniline **(37)**	3.63 (A);	3.78 (B)	258
N,N-Bis(2-thiocyanatoethyl)aniline **(40)**	14.79 (A)		249
N,N-Bis(2-nitratoethyl)aniline **(41)**		10.65 (B)	260
4-Phenylmorpholine **(45)**	6.76 (A);	7.21 (B)	260
DMSO	2.73 (A);	2.45 (B)	cut off 250

5.4.3.2 HPLC conditions for analysis of hydrolysis products from mustards (5), (10) - (12) and (58)

The identity and quantity of hydrolysis products from the mustard substrate were usually determined by reverse-phase HPLC using the conditions and gradient programmes summarised in ***Table 5.3.*** This table also lists the retention times for several authentic mustards **(5), (10), (11), (12)** and **(58)**, possible hydrolyse products **(37), (45), (59), (60), (61), (62), (64), (65), (66), (86), (115)** and **(116)**, and some intermediate (half hydrolysed) mustards **(67), (68), (69)** and **(117).**

Figure 5.2 shows typical HPLC chromatograms for the hydrolysis of mustards **(10) (11)** and **(12)** in aqueous phosphate buffer/DMSO (3%,v/v) at pH 7.4 and 37°C at t=0 min (a), after 30 min (b) and after 80 min (c) of reaction, run under the conditions described in ***Table 5.3.***

Table 5.3

HPLC conditions and retention times for authentic materials used in product analysis of the mustards (5), (10), (11), (12) and (58)

Column: C_8-APEX II OCTYL 5µ; 25 cm x 4.6 mm i.d.

Temperature: 30°C

Flow rate: 1 cm^3/min

Detection: UV 249 nm

Injection : 20 µl

Eluent: Two component gradient system using Solution A: aqueous. KH_2PO_4 buffer (0.05M, pH 2.6) with 1%ACN* and

Solution B: aqueous KH_2PO_4 buffer (0.05M, pH 2.6) with 60% ACN*

Gradient program: Programme 1 and 2 as shown:

Programme 1 (P_1)			Programme 2 (P_2)		
Time (min)	%A	%B	Time (min)	% A	% B
0	95	5	0	90	10
5	95	5	3.5	0	100
20	10	90			
24	0	100			

* Solution A prepared from KH_2PO_4 (1M, 50cm^3) + ACN (10cm^3) + H_3PO_4 (1cm^3) diluted with H_2O to 1l

*Solution B prepared from KH_2PO_4 (1M, 50cm^3) + ACN (600cm^3) + H_3PO_4 (1cm^3) diluted with H_2O to 1l

Table 5.3 (continued)

Compound	Retention time (Rf) (min)/ (P_1 or P_2)		λ max in HPLC eluent
N,N-Bis(2-chloroethyl)aniline **(5)**		20.47(P_2)	256
N,N-Bis(2-chloroethyl)-4-amino-aniline **(10)**	20.61(P_1)		258
N-[4-[*N'*,*N'*-Bis(2-chloroethyl)aminophenyl]acetamide **(11)**	24.77(P_1)	10.63(P_2)	276
N-[4-[*N'*,*N'*-Bis(2-chloroethyl)amino]phenyl] trifluoroacetamide **(12)**	29.80(P_1)		290
N-[4-[Bis*N'*,*N'*-(2-acetyloxy)ethyl]amino] phenyl] acetamide **(58)**	19.91(P_1)		276
N-[4-[*N'*,*N'*-Bis(2-hydroxyethyl)amino] phenyl] acetamide **(59)**	8.63(P_1)		249
N,N-Bis(2-hydroxyethyl)aniline **(37)**		4.92(P_2)	258
N,N-Bis(2-hydroxyethyl)-4-amino-aniline **(66)**	4.20(P_1)		258
4-Morpholinoaniline **(61)**	12.18(P_1)		246
4-Acetamidophenol **(62)**	11.06(P_1)		246
4-Phenylmorpholine **(45)**	19.69(P_1)		242
4-Morpholinophenylacetamide **(60)**	15.16(P_1)		254
N-[4-[*N'*,*N'*-Bis(2-hydroxyethyl)amino] phenyl] trifluoroacetamide **(65)**	17.75(P_1)		290, 248
4-Aminophenol **(64)**	12.49(P_1)		249,270
4-Phenylenediamine **(86)**	2.74(P_1)		239
Acetanilide **(115)**	16.95(P_1)		244
Morpholine **(116)**	16.87(P_1)		
DMSO	2.95(P_1)	2.98(P_2)	cut off 250
N-[4-[*N'*-(2-Acetyloxyethyl)-*N'*-(2-hydroxy-ethyl) aminophenyl] acetamide **(70)**	15.16(P_1)		276
N-[4-[*N'*-(2-Chloroethyl)-*N'*-(2-hydroxyethyl) aminophenyl] acetamide **(67)**	17.87(P_1)		278
N-[4-[*N'*-(2-Chloroethyl)-*N'*-(2-hydroxyethyl) aminophenyl]trifluoroacetamide **(68)**	23.61(P_1)		297
N-(2-Chloroethyl)-*N*-(2-hyroxyethyl)aniline **(117)**		10.21(P_2)	263
N-(2-Chloroethyl)-*N*-(2-hyroxyethyl)-4-amino-aniline **(69)**	14.64(P_1)		

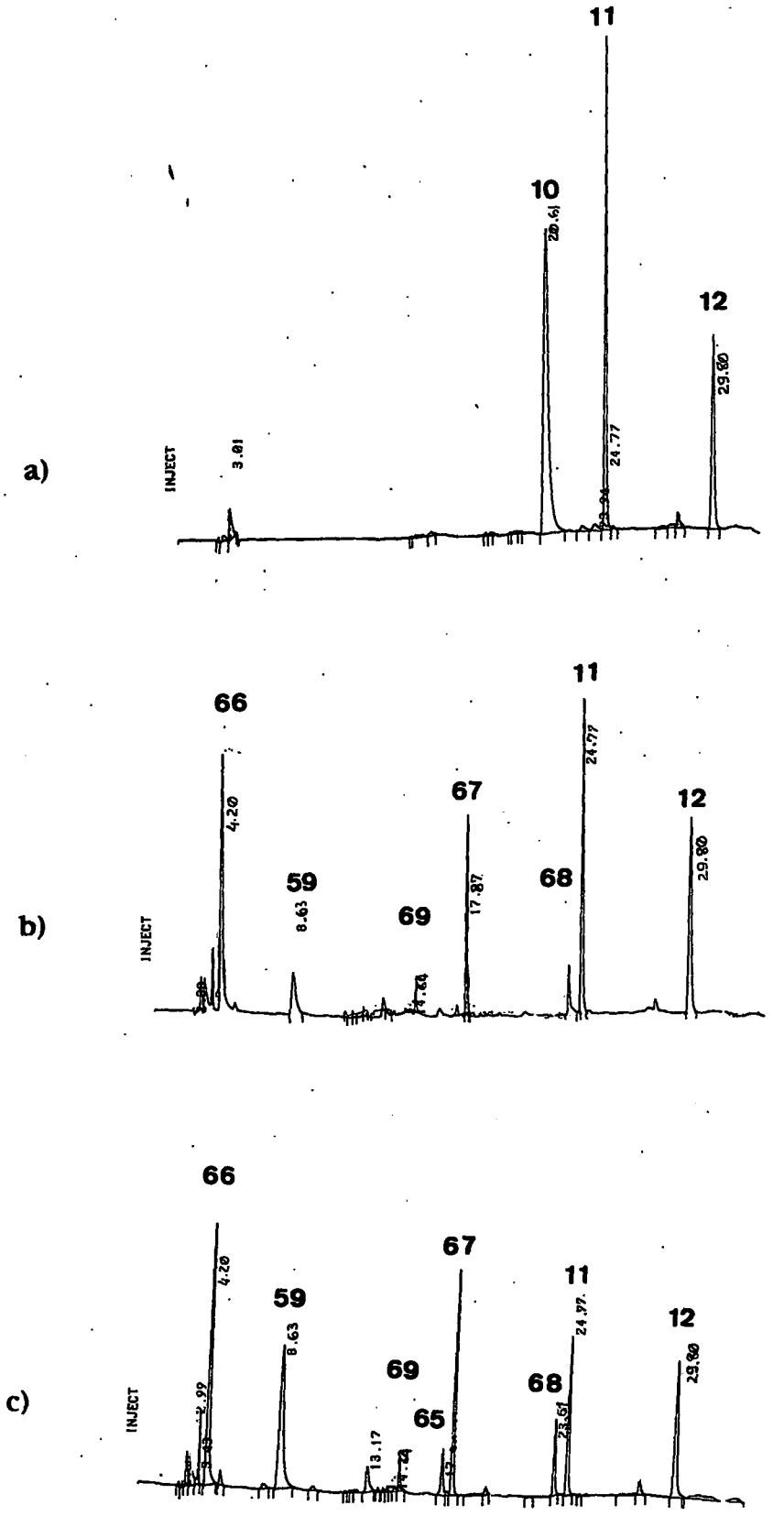


Figure 5.2 *HPLC chromatograms of mustards (10), (11) and (12) hydrolyses in aqueous phosphate buffer/DMSO (3%,v/v) at pH 7.4 and 37°C: a) t=0 min b) t=30 min c) t=80 min*

5.4.3.2.1 Calibration curves for standards

To quantify the reaction products from the hydrolysis of mustards **(5)**, **(10)**, **(11)** and **(12)**, calibration curves were generated for the substrates **(11)** and **(12)** and major final products (**(59)**, **(60)** and **(65)**).

Generally, standard solutions of the relevant compound in H_20/DMSO (7:3,v/v) obtained by dilution of a gravimetrically prepared stock solution, were assayed and quantitated by either peak area or peak height. The HPLC conditions used are those described in ***Table 5.3***. All the calibration curves were linear and similar to the one shown in ***Figures 5.3*** for compound **(60)**. The linear coefficients are summarised in ***Table 5.4***.

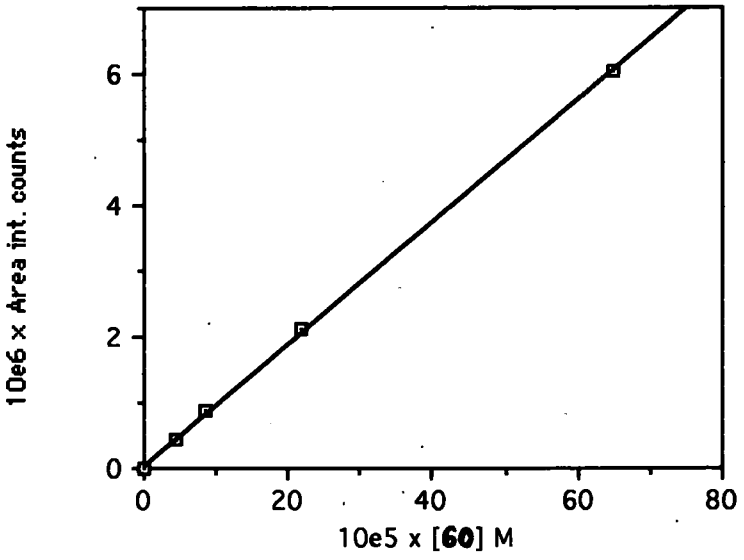


Figure 5.3 *HPLC calibration curve for authentic* ***(60)***

Table 5.4

Coefficients for HPLC calibration curves for authentic ***(11)***, ***(12)***, ***(59)***, ***(60)*** *and* ***(65)***; *λ=249 nm, eluent: aqueous KH_2PO4 buffer (pH 2.6)*

Compound	10^4 x Linear coefficient
N-[4-[*N'*,*N'*-Bis(2-chloroethyl)aminophenyl] acetamide **(11)**	2.81
N-[4-[*N'*,*N'*-Bis(2-chloroethyl)amino]phenyl] trifluoroacetamide **(12)**	1.21
N-[4-[*N'*,*N'*-Bis(2-hydroxyethyl)amino]phenyl] acetamide **(59)**	3.78
4-Morpholinophenylacetamide **(60)**	9.25
N-[4-[*N'*,*N'*-Bis(2-hydroxyethyl)amino]phenyl] trifluoroacetamide **(65)**	3.91

5.4.3.3 Hydrolysis of mustards (5), (10) - (12) and (58) in aqueous phosphate buffer/ /DMSO (3%, v/v) at pH 7.4 and 37°C

Generally, 150 μl of mustard (*ca.* $3x10^{-2}$M) in DMSO was injected into a reaction vessel containing aqueous phosphate buffer ($1x10^{-3}$M) at pH 7.4 and DMSO to give a final concentration of 3% (v/v) and a total volume of 30 cm^3. The concentration of each mustard was *ca.* $1.5x10^{-4}$ M. The reaction vessel was in a thermostatted bath at 37°C and the solution was stirred continuously. Aliquots (20μl) of the reaction solution were analysed by HPLC under the conditions summarized in ***Table 5.3.*** The elution gradient for the assay of mustard (5) (programme 2), was different from that for (10), (11), (12) and (58) (programme 1) so, mustard (5) was hydrolysed in a separate experiment simultaneously with mustard (11), which acted as a control. The disappearance of the substrate mustard was followed because several labile intermediates which reacted further, were evident. For all the mustards both the starting materials and reaction products were assayed.

5.4.3.4 Hydrolysis of mustards (10) - (12) and (58) in aqueous DMSO (7:3, v/v) at pH 2, 12 or 13 and 37°C or 50°C

Similar to the procedure described above, 50 μl of the mustard (*ca.* $9x10^{-2}$ M) in DMSO, was injected into a reaction vessel containing aqueous DMSO (7:3, v/v) at pH 2, 12, or 13, adjusted by addition of either HCl (1M) or NaOH (1M) (30 cm^3). The final concentration of the mustard was *ca.* $1.5x10^{-4}$ M. The reaction vessel was in a thermostatted bath at 37°C (or 50°C for mustard (58))and the reaction solution was stirred continuously. Aliquots (20μl) of the reaction mixture were analysed by HPLC under the conditions outlined in ***Table 5.3.*** Both the disappearance of substrates and the formation of products was assayed and identified major products were quantified from calibration curves generated with authentic compounds.

5.4.4 Kinetics of mustard (5) and (10) - (12) hydrolysis in aqueous phosphate buffer followed by ion exchange chromatography

The same general procedure to that described in Section 5.4.3.3 was followed. Thus, 150 μl of each mustard (*ca.* $3x10^{-2}$ M) in DMSO was injected into a reaction vessel containing aqueous phosphate buffer ($1x10^{-3}$M) at pH 7.4 and DMSO (3%,v/v) thermostatted at 37°C. The total volume was 30 cm^3 and the concentration of the substrate *ca.* $1.5x10^{-4}$M. Due to practical problems, it was not possible to stir the reaction mixture. Aliquots (20 μl) of the reaction mixture were analysed for Cl^- by ion exchange chromatography under the conditions described in ***Table 5.5.*** The retention times of Cl^- and $HPO_4^=$ (buffer) from injection of

standard solutions of KCl ($1x10^{-3}M$) and the phosphate buffer used for the hydrolysis reaction ($1x10^{-3}M$) are also listed.

Table 5.5

Conditions for analysis by ion exchange chromatography and retention times obtained for authentic phosphate buffer and Cl^-

Column: Ion chromatography 300 I.C. (VYDAC); 0.46 x 5 cm
Temperature: 30°C
Eluent: 2% ACN in 0.25mM phthalic acid pH 7.0, adjusted with triethylamine
Flow rate: $2cm^3/min$
Detector: UV 249nm
Injection: 20 µl

Ion	Rf (min)
Cl^-	11.8
$HPO_4^=$	8.2

5.4.5 Isolation and identification of hydrolysis products for mustards (11) and (12) by GC and GC-MS

Substrate (11) (20 mg, 0.072 mmol) or (12) (23 mg, 0.072 mmol) in aqueous DMSO (7:3,v/v) (25 cm^3) at pH 2, 7and 12 [pH adjusted with HCl (1M) or NaOH (1M)] was heated at 37°C, with stirring, for 24 h. Where necessary, the reaction mixture was neutralized to pH 7 with HCl (1M) or NaOH (1M) and immediately freeze-dried. The residue was extracted into ethyl acetate (20 cm^3), washed with water (15 cm^3) and dried over anhyd. $MgSO_4$. It was then analysed by GC, GC-MS, MS (FAB) and MS (EI). The solvents used to dissolve the samples were ACN for the GC and GC-MS and 2-thiodiethanol for MS(FAB).

The GC analysis was done against authentic 4-morpholinoaniline (61), 4-acetamidophenol (62), 4-phenylmorpholine (45), diethanolamine (63), and 4-morpholinophenylacetamide (60). Identification was confirmed by spiking the reaction mixture with the authentic compound.

In *Table 5.6*, both the GC and GC-MS conditions are summarised together with retention times for authentic compounds.

Table 5.6

Conditions for GC and GC-MS analyses and retention times for authentic compounds involved in identification of hydrolysis products for mustards (11) and (12)

GC conditions: Capillary BP5 column: 25m x 0.53mm;
Detector: N/P, Temp: 276°C
Injector temp 275°C
Oven programme: 90°C (2min) to 300°C (1h) (10°C/min)

Capillary BP20 column : 25m x 0.53mm
Detector: FID, Temp: 276°C
Injector temp: 275°C
Oven programme: 90°C (1min) to 230°C (1h) (16°C/min)

GC-MS conditions: Capillary BP20 column : 12m x 0.53mm;
Oven program: 80°C (2min) to 240°C at rate (30°C/min)

Compound		GC Rf (min) Column: BP5 / BP20
N-[4-[*N'*,*N'*-Bis(2-chloroethyl) aminophenyl]acetamide	**(11)**	17.5 / -
N-[4-[*N'*,*N'*-Bis(2-chloroethyl) amino]phenyl]trifluoroacetamide	**(12)**	23.2 / -
4-Phenylmorpholine	**(45)**	8.4 / -
4-Morpholinophenylacetamide	**(60)**	18.82 / 22.9
4-Morpholinoaniline	**(61)**	10.9 / -
4-Acetamidophenol	**(62)**	10.3 / -
Diethanolamine	**(63)**	3.3 / 5.89

5.4.6 Decomposition of *N'*-acetyl carbamate (26)

5.4.6.1 By UV-visible spectrophotometry

In the usual procedure, an aliquot (10 µl) of the N'-acetyl carbamate **(26)** in DMSO (1.2 x 10^{-2}M) was injected into a cuvette containing either aqueous DMSO (7:3, v/v) (3 cm^3), at pH 2, 7 or 12 (adjusted with NaOH 1M or HCl 1M), or the organic solvent. The final concentration of substrate was 4x10^{-5}M. The cuvette was kept at either 25°C for 24h, or 1h at 25°C then at 70°C for 48h, by water circulated through the cuvette holder.

The UV/visible spectra of the cell contents were recorded (240-500 nm) immediately after injection of the substrate into the cuvette, and at timed intervals (15 min during the first 75 min; 60 min during the next 12h and after 24h and 48h).

5.4.6.2 By analytical HPLC

Usually, an aliquot (10 μl) of the N'-acetyl carbamate **(26)** in DMSO ($1.2x10^{-2}$ M), was injected into a volumetric flask containing either aqueous DMSO (7:3, v/v, 3 cm^3), at pH 2, 7 or 12 (adjusted with either NaOH 1M or HCl 1M) or the organic solvent. The final concentration of substrate was *ca.* $4x10^{-5}$M. The reaction flask was kept at 37°C in a thermostatted water bath. At timed intervals, aliquots (20 μl) of the reaction solution were taken and analysed by reverse-phase HPLC. The HPLC parameters for these assays are summarised in ***Table 5.7.***

The products were identified by their retention times (Rf) relative to those for authentic compounds, summarised in ***Table 5.7.*** The relative areas of the 4-(*N*'-acetyl)amino carbamate substrate **(26)** and products were obtained from the integrator output.

To quantify the reaction products, calibration curves were generated for the substrate and major final products. Generally, standard solutions of the relevant compound in H_2O/DMSO (7:3,v/v) obtained by dilution of a gravimetrically prepared stock solution, were assayed and quantitated by peak area. The HPLC conditions used are those described in ***Table 5.7.*** All the calibration curves were linear and the linear coefficients are summarised in ***Table 5.8.***

A typical chromatogram for decomposition of *N*'-acetyl carbamate **(26)** (Rf=24.01 min) in aqueous DMSO at pH 12 after 48 h, showing formation of 4-nitroaniline **(92)** (Rf=19.20 min), 4-aminobenzyl alcohol **(80)** (Rf=2.98 min) and 4-acetamidebenzylalcohol **(96)** (Rf=13.58 min), is shown in ***Figure 5.4.***

Table 5.7

HPLC conditions and retention times for authentic compounds involved in decomposition of carbamate (26)

Column: C_{18}-APEX II 5μ; 25 cm x 4.6mm i.d.
Temperature: 30°C
Flow rate: 1 cm^3/min
Detection : UV 249nm
Injection: 20 μl in auto injection or manually
Eluent: Two component gradient using: Solution A - 1% (v/v)

ACN in 0.05M aqueous KH_2PO_4 (pH 2.6)* and

Solution B- 60% ACN in 0.05M aqueous KH_2PO_4 (pH 2.6)*

Gradient program:

Time (min)	%A	%B
0	90	10
3.5	0	100
30	90	10

* Solution A prepared from KH_2PO_4 (1M, $50cm^3$), ACN ($10cm^3$) and H_3PO_4 (85%, p/v,$1cm^3$) diluted to 1l

*Solution B prepared from KH_2PO_4 (1M, $50cm^3$), ACN ($600cm^3$) and H_3PO_4 (85%, p/v, $1cm^3$) diluted to 1l.

Compound		Rf (min)	λ (nm) / log ε at HPLC eluent
4-(*N*'-Acetylamino) carbamate	**(26)**	24.01	325 / 1.23 249 / 1.26
4-Aminobenzyl alcohol	**(80)**	2.98	249 / 1.13
4-Nitroaniline	**(92)**	19.20	381 / 1.06 249 / 0.49
4-(*N*-Acetylamino)benzyl alcohol	**(96)**	13.58	246 / 1.15 249 / 1.17

Table 5.8

Coefficients for HPLC calibration curves for authentic compounds involved in decomposition of (26): λ=249 nm, eluent: aqueous KH_2PO4 buffer (pH 2.6)

Compound		10^4 x Linear coefficient
4-(*N*'-Acetylamino) carbamate	**(26)**	1.84
4-Aminobenzyl alcohol	**(80)**	1.36
4-Nitroaniline	**(92)**	0.31
4-(*N*-Acetylamino)benzyl alcohol	**(96)**	1.49

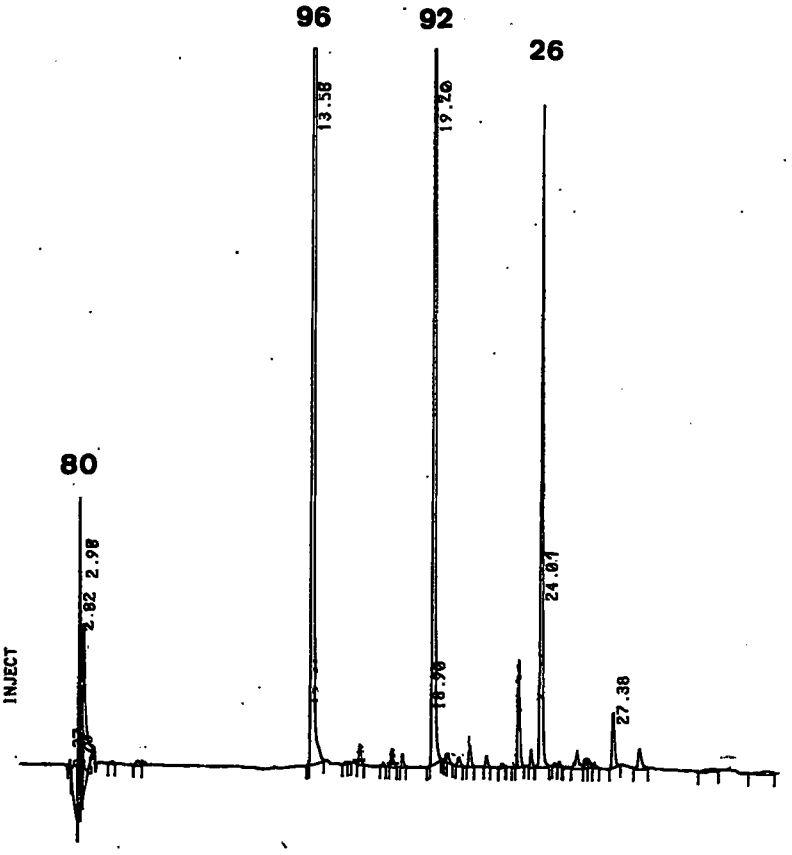


Figure 5.4 HPLC chromatogram for the hydrolysis of N'acetyl carbamate (26) in aqueous DMSO (7:3,v/v) at pH 12 and 37°C after 48h: $[26]_{init.}$ ca. $4x10^{-5}M$; λ=249 nm

5.4.6.3 Hydrolysis by the enzyme acylarylamidase

5.4.6.3.1 UV-visible spectrophotometric studies of (26) and (103) hydrolyses

A solution of compound **(103)** or **(26)** in DMSO (15 µl, $1x10^{-3}M$) was injected into a solution of tris/HCl buffer (0.1M, pH 8.6, 1470 µl) contained in the cuvette of the spectrophotome ter thermostatted at 37°C. Subsequently, either acylarylamidase enzyme E.C. 3.5.1.13 ($1.8x10^{-2}$ unit/cm^3) in tris/HCl (0.1M, pH 8.6, 15 µl), or simply tris/HCl (0.1M, 15 µl) without enzyme, was added to the cell contents. The final concentration of **(103)** or **(26)** was $1x10^{-5}M$ and the amount of enzyme was $2.7x10^{-4}$ units, i.e. $18x10^{-5}$ units/cm^3. The cell contents were monitored between 250 and 500 nm at timed intervals.

5.4.6.3.2 HPLC studies of (26) hydrolysis

To a solution of compound **(26)** in DMSO (15 µl, $1x10^{-3}$M) in tris/HCl (0.1M, pH 8.6, 1470 µl), at 37°C was added acylarylamidase enzyme E.C. 3.5.1.13 ($1.8x10^{-2}$unit/cm^3) in tris/HCl (0.1M, pH 8.6, 15 µl). Aliquots of the reaction solution were taken just before addition of the enzyme, immediately after addition of the enzyme, and after 3 min and 15 min, were analysed by HPLC against authentic compounds. The HPLC conditions and retention times were the same as those described in Section 5.4.6.2.

5.4.7 Decomposition of carbamate (74)

5.4.7.1 General procedure

Carbamate **(74)** (4-8 mg) dissolved in a mixture of DMSO and chloroform in a ratio of (3:7, v/v) (2 cm^3), was heated in a thermostatted bath at 65°C over 48h.

At timed intervals (2h, 5h, 10h, and 48h) the reaction mixture was examined by ^{1}H-NMR. After 5-6h, a precipitate was visible in the NMR tube.

Aliquots (5 µl) of the reaction mixture of compound **(74)** at timed intervals were also analysed by GC using either BP5 or BP20 columns and either FID or N,P detectors, against authentic 4-nitroaniline **(92)**, 4-aminobenzylalcohol **(80)** and p-toluidine **(89)**.

Aliquots (5 µl) of the reaction mixture after 2h and 48h of reaction were also analysed by GC-MS on a BP5 column against authentic compounds. Conditions and retention times for the GC and GC-MS assays are given in ***Table 5.9***.

Table 5.9

Conditions and retention times for GC and GC-MS assay of carbamate (74)

GC conditions:	Condition A)	Capillary BP5 column: 12.5m
		Oven program: 90°C (2min) to 300°C(2h)
		rate 10°C/min
		Detector: Rb bead FID, Temp 250°C,
		Injection: On column, 5µl
	Condition B)	Capillary BP20 column: 12.5m
		Oven program: 90°C (1min) to 250°C (3h)
		rate 16°C/min
		Detector: FID Temp 250°C

Injection : Temp 200°C, 5μl

GC-MS conditions:

Capillary BP5 column:12.5m

Oven program: 90°C (2min) to 300°C(20min)

rate 10°C/min

Injection: On column, 2μl

Compound	GC column / Rf (min)
N-acylcarbamate **(74)**	BP5 / not seen
	BP20 / not seen
4-Aminobenzyl alcohol **(80)**	BP5 / 8.26
	BP20 / not seen
4-Toluidine **(89)**	BP5 / 3.85
	BP20 / 4.12
Nitroaniline **(92)**	BP5 / 11.91
	BP20 / 13.0

5.4.7.2 Decomposition in the presence of LiI

The carbamate **(74)** (5.4 mg, 0.019 mmol) was dissolved at room temperature in DMSO/$CHCl_3$ (3:7, v/v) (1.1 cm^3). Lithium iodide (2.5 mg, 0.019 mmol) was added, and the solution was heated at 65°C for 3h. Silica tlc using ether as eluent showed complete disappearance of the starting material after 2h. The reaction mixture was concentrated under vacuum, the residue dissolved in ethyl acetate, applied to a preparative silica tlc plate which was eluted with DCM. Three major fractions were obtained showing Rf=0.6 (Fraction 1), Rf=0.4 (Fraction 2) and Rf=0.2 (Fraction 3). These were removed from the plate, extracted with DCM, the solvent vacuum evaporated and the three residues obtained were analysed by NMR and MS(FAB)/MS(EI). Fraction 1 and 2 were also analysed by GC (BP5 column under the same conditions described in Section 5.4.7.1).

5.4.8 Hydrogenolysis of *N'*-CBZ carbamates (75) and (76) and carbamate (104)

5.4.8.1 General procedure

Usually, the carbamate (1 mmol) was dissolved in either acid-washed, freshly distilled ethyl acetate or absolute ethanol (100 cm^3) with (15%, w/w) palladium on charcoal (0.1 mmol) and stirred under hydrogen at room temperature. The reaction was monitored by silica tlc using ether/n-hexane (4:1, v/v) eluent for compound (75), and by GC for compounds (76) and (104). For (76) and (104) the reaction products were identified by GC-MS of the reaction mixture after a simple filtration. For compound (75) the final products were identified by ^{1}H-NMR and MS of the residue obtained after filtration of the solution and vacuum evaporation of the solvent at low temperature.

5.4.8.1.1 Product identification for carbamate (76)

The products were identified by GC retention times against authentic compounds, including spiking of the reaction solution. Their identity was confirmed by GC-MS and interpretation of the MS fragmentation spectra.

The GC and GC-MS conditions and related retention times of the products of hydrogenolysis for carbamate (76) are summarised in *Table 5.10.*

Table 5.10

GC and GC-MS conditions and retention times for compounds involved in hydrogenolysis of carbamate (76)

GC conditions:	Capillary BP20 column: 12.5m
	Detector: FID, Temp 276°C
	Injector temp: 260°C
	Oven program: 80°C (2min) to 200°C (15min) at rate (10°C/min); 200 to 220°C rate (10°C/min)
	Injection : 2 µl
GC-MS conditions:	Capillary BP20 column: 12.5m
	Injector Temp: 260°C
	Oven program: 80°C (2min) to 220°C (20min) at rate (10°C/min), 220 to 240°C at rate (30°C/min)
	Injection : 2 µl

Compound	GC Rf (min)
N-(4-trifluoromethylphenyl)carbamate **(76)**	47.25
4-(*N*-Benzyloxycarbonylamino)benzyl alcohol **(85)**	18.11
4-Aminobenzotrifluoride **(87)**	13.91
Benzylalcohol **(90)**	12.30

5.4.8.1.2 Product identification for carbamate (104)

Table 5.11 shows the GC and GC-MS conditions as well as the GC retention times for the products involved in hydrogenolysis of **(104)**.

Table 5.11

GC and GC-MS conditions and retention times for the hydrogenolysis of ***(104)***

GC conditions: Capillary BP20 column: 25m
Detector Temp: 276°C;
Injector temp: 260°C
Oven programme: 80°C (2min) to 200°C (15min) rate (10°C/min) and to 220°C (6h) rate (10°C/min)
Injection : 10 µl

GC-MS conditions: Capillary BP20 column: 12m
Oven program: 80°C(2min) to 240°C rate (30°C/min)
Injector temp: 250°C
Injection : 2 µl sample in EtOH

Compound	GC Rf (min)
Aniline **(106)**	11.79
4-Aminobenzyl alcohol **(80)**	not seen
4-Toluidine **(89)**	12.66
4-Nitroaniline **(92)**	62.53
4-Nitrobenzyl *N*-phenyl carbamate **(104)**	66.12
4-Nitrobenzylalcohol **(105)**	45.38
4-Nitrotoluene **(107)**	13.45

5.4.9 Hydrolysis of alcohol (91)

4-(*N*-Triphenylmethylamino)benzyl alcohol **(91)** (7.3 g, 20 mmol) in acetone (25 cm^3) and HCl (1M, 0.5 cm^3) was stirred at room temperature for 2h. The solution was neutralized with aqueous satd. solution of $NaHCO_3$ to pH 7.5 and extracted into DCM (45 cm^3). The extract was washed with water (25 cm^3), dried over anhyd. $MgSO_4$ and the solvent vacuum evaporated. The product was identified by ^1H NMR and MS(FAB^+).

5.4.10 Hydrolysis of carbamate (113)

Ethyl *N*-(4-nitrophenyl) carbamate **(113)** in DMSO (10 µl,14.5mM) was injected in a UV cuvette containing aqueous DMSO (9:1,v/v) (3 cm^3) at pH 2, 7 and 12 (adjusted with either HCl or NaOH) thermostatted at 37°C. The concentration of **(113)** in the cuvette was $4.8x10^{-5}$M. The reaction was monitored by recording the spectrum between 200-500 nm at timed intervals.

5.4.11 Hydrolysis of ester (95)

A solution of 4-(*N*-acetylamino)benzyl methyl ester **(95)** (0.69 g, 3.3 mmol) in ethanol/H_2O (7:20, v/v) pH 12 (adjusted with NaOH 1M, 4.5 cm^3) was reacted at 40°C for 6h. At timed intervals, aliquots (100 µl), diluted with H_2O (1 ml) and neutralized with 0.1M HCl, were assayed by HPLC (10 µl).

Hydrolysis of compound **(95)** was monitored by following the disappearance of the peak at Rf= 4.18 min **(95)**, and the appearance of a peak at Rf= 2.78 min [4-(*N*-acetylamino)benzyl alcohol **(96)**] using the HPLC conditions described in ***Table 5.12***.

Table 5.12
HPLC conditions involved in hydrolysis of ester (95)

Column: C8-Apex II octyl; 25cm x 4.6mm i.d.
Eluent Isocratic: aqueous phosphate (0.02M)/CH_3CN (3:2,v/v)
Flow rate: 1 cm^3/min
Detector: UV 263 nm
Injection: 10µl

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Appendix

POTENTIAL PRODRUGS FOR ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY (ADEPT)*

John Baer[1], Azadeh Freeman[1], Gordon T. Rogers[1], Philip J. Burke[1], Edward S. Newlands[1], J.N. Iley[2], M.T.T. Pamplona[2], Brian C. Challis[2] & Kenneth D. Bagshawe[1].

[1]Cancer Research Campaign Laboratories, Department of Medical Oncology, Charing Cross Hospital, London W6 8RF, UK and
[2]Dept. of Chemistry, Open University, Milton Keynes, MK7 6AA, UK.

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Summary

The cytotoxicity of several N-acetylated aryl nitrogen mustard prodrugs was assessed in LS174T human colorectal cells. HPLC analysis showed that the N-acetylated 4-aminoaryl mustard prodrug was a substrate for a bacterial acylarylamidase enzyme (E.C. No. 3.5.1.13.) with release of the aminoaryl ustard. The N-acetylated prodrugs were nearly as toxic as the parent drug in LS174T and also JAR choriocarcinoma cells. There was no evidence of conversion of prodrug to the parent drug by the cells. A series of model compounds, synthesised to optimise the rate of turnover, showed that relatively few N-acyl groups are substrates for the enzyme with the N-chloroacetyl analogue being most reactive.

Introduction

Monoclonal antibodies have been extensively studied to assess their potential use in cancer chemotherapy, carrying tumour-directed poisons such as radiolabel[8] or powerful toxins[6] or as agents to expose the tumour cells to attack from the body's immune system[12]. These therapies are limited to the once-only effect of the antibody or toxin at the tumour site and require a high level of homogeneity in tumour cell antigen expression for effective localisation and treatment. Repeated administration is limited by the immunogenicity of non-human antibodies.

An alternative means of treatment is antibody-directed enzyme prodrug therapy (ADEPT). A conjugate is prepared by covalently linking an enzyme to a tumour-directed monoclonal antibody. Once localised, the conjugate can activate a relatively non-toxic prodrug to a cytotoxic drug at the tumour site[1], thus avoiding systemic toxicity. Multiple doses of the prodrug could be

administered during the time that conjugate remains active at the tumour site; active drug formed by the conjugate would be capable of reaching cells in tumour masses that are inaccessible to antibodies. An initial study, in this Department, using a carboxypeptidase enzyme-antibody conjugate and a glutamated benzoic acid mustard prodrug directed against choriocarcinoma permanently eliminated the xenograft in nude mice[2]. This system is currently undergoing clinical trials. Another group of workers[11], using etoposide phosphate as prodrug, has shown that animals treated with alkaline phosphatase conjugates have a much better anti-tumour response to tumour-directed than non-localised conjugate, indicating that this effect is not simply due to non-specific slow release of cytotoxic species.

The present work describes attempts to design more reactive active species and to maximise the difference in toxicity between prodrug and active species. Acylarylamidase, an enzyme of bacterial origin, was selected to avoid activation of prodrug, 4-amidoaryl mustards, by endogenous enzymes *in vivo*. The enzyme cleaves the electron-withdrawing N-acyl substituent (Figure 1) and releases the more reactive parent drug, 4-aminoaryl mustard. Alkylating agents of the nitrogen mustard type were chosen because their mode of action is non-cell cycle dependent and they tend not to induce high levels of resistance in cells[13].

Materials and Methods

Acylarylamidase enzyme (E.C. No. 3.5.1.13.) was a gift from Dr Roger Sherwood, Division of Biotechnology, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, UK.

All chemicals were Analar grade and supplied by BDH (Poole, Dorset, UK).

Cell culture materials were supplied by Flow Laboratories,

(Richmond, Hertfordshire, UK).

Cell Culture

Human LS174T colorectal or JAR choriocarcinoma cells were grown in a 5% CO_2 atmosphere at 37°C. The growth medium was DMEM containing 10% foetal calf serum supplemented with 2mM glutamine and penicillin/streptomycin (100 units/ml and 100ug/ml respectively). Cells were plated out at a density of 5 x 10^4 cells/ml in 2ml medium in 6 well plates. After overnight incubation to allow their attachment to plastic, the cells were treated with the drug for 1 hour and then rinsed with fresh medium. Drug solutions were prepared immediately before addition to the cells. Appropriate solvent controls were included. After 7 days (LS174T) or 5 days (JAR) incubation the cells were trypsinised and counted by the trypan blue exclusion method. IC_{50} and IC_{90} values were estimated by non-linear regression software, PCNONLIN (SCI Software, Lexington, Kentucky, USA).

HPLC

HPLC was performed on a Waters automated system containing a 6000A pump, 490 UV detector set at 278nm and 712B WISP autosampler, injection volume 10ul. A 5u Apex Octyl column (25 x 0.4cm) was used at a temperature of 40°C. All solvents were filtered through 0.45um filters. The mobile phase contained 60% acetonitrile, 40% 0.05M ammonium acetate pH 4.0 at 1.2ml/min. HPLC samples from enzyme assays were prepared by adding an equal volume of acetonitrile to precipitate protein. The supernatant was injected onto the HPLC following centrifugation.

The resulting chromatograms are shown in Figure 2. The assay was linear ($r>0.98$) up to a concentration of 1mM. Intra-assay precision was 2.1% for the N-trifluoroacetyl analogue (n=5).

Samples containing 4-aminoaryl mustard ($T_{\frac{1}{2}}$ = 67 min for an aqueous sample prepared as above) were injected immediately.

Synthesis

N,N-Bis-(2-chloroethyl)-4-nitrosoaniline (Compound 5): Aniline was converted in 71% yield using ethylene oxide to its N,N-bis-(2-hydroxyethyl) derivative[10], treatment of which with tosyl chloride gave the bis-tosylate in 81% yield. Heating the bis-tosylate with $CaCl_2$ in 2-ethoxyethanol at 120°C furnished N,N-bis-(2-chloroethyl) aniline (60%) [4] and this was converted to N,N-bis-(2-chloroethyl)-4-nitrosoaniline in 78% yield using nitrous acid[5]. Recrystallisation from ether yielded green plates: m.p. 79-80°C (literature m.p.[3] 79-80°C); ν_{max} (nujol) 1600, 1500, 1380, 1100, 820, 710 cm^{-1}; s_H ($(CD_3)_2CO$) 4.0 (8H, d.t.), 7.0 (2H,d,J=9.5Hz), 7.8 (2H,d,J=9.5Hz); m/z 247 (M^+).

N-[4-(N',N'-Bis-(2-chloroethyl)amino)phenyl]acetamide (Compound 1): The 4-nitrosoaniline derivative (Compound 5) was reduced using hydrogen with 15% Pd/C catalyst (0.2 equiv. w/w) to N,N-bis-(2-chloroethyl)-4-phenylene diamine, which was converted immediately to the hydrochloride salt in a crude yield of 81%. This crude salt was acetylated with acetic anhydride/triethylamine[10], and N-[4-(N',N'-bis-(2-chloroethyl)amino)phenyl]acetamide was obtained in 25% yield after purification by column chromatography (silica) and recrystallisation from ether/n-hexane: m.p. 120°C (literature m.p.[5] 124-126°C); ν_{max} (KBr) 3300 (N-H), 1650 (C=O), 1540, 1520, 1330, 1140, 820 cm^{-1}; s_H ($(CD_3)_2CO$) 2.1 (3H,s), 3.72 (8H,s), 6.71 (2H,d,J=9Hz), 7.49 (2H,d,J=9Hz), 8.8 (1H,br. ex.); m/z 274 (M^+), 225 ($M-CH_2Cl$); (Found: C, 52.3; H, 5.9; N, 9.9%. Calc. for $C_{12}H_{16}Cl_2N_2O$: C, 52.4; H, 5.9; N, 10.2%).

N-[4-(N',N'-Bis-(2-chloroethyl)amino)phenyl]trifluoroacetamide (Compound 2): As for compound 1 using trifluoroacetic anhydride/triethylamine. Purification by column chromatography (silica) and recrystallisation from ether/n-hexane gave N-[4-(N',N'-Bis-(2-chloroethyl)amino)phenyl]trifluoroacetamide in 20% yield: m.p. 101-1033C (literature m.p.[5] 109-1103C); ν_{max} (KBr) 3300 (NH), 1730, 1700 (C=O), 1620, 1600, 1550, 1520, 1360, 1150 and 820 cm^{-1}; s_H ($(CD_3)_2CO$) 3.8 (8H,s), 6.83 (2H,d,J=9Hz), 7.59 (2H,d,J=9Hz), 10.1 (1H,br. ex.);m/z 328 (M^+), 279 ($M-CH_2Cl$); (Found C, 44.1; H, 4.1; N, 8.4%). Calc. for $C_{12}H_{13}Cl_2F_3N_2O$: C, 43.8; H, 4.0; N, 8.5%).

N-[4-(N',N'-Bis-(2-chloroethyl)amino)phenyl]chloroacetamide (Compound 3): This was prepared using a standard method[10]: s_H ($CDCl_3$) 3.62 (4H,t), 3.77 (4H, t), 4.19 (2H,s), 6.67 (2H,d), 7.40 (2H,d).

N-[4-(N',N'-Bis-(2-acetyloxyethyl)amino)phenyl]acetamide (Compound 4): N,N-Bis-(2-hydroxyethyl)-4-phenylene diamine obtained as for the bis-(2-chloroethyl) analogue above was stirred with excess (20 equiv. w/w) acetic anhydride/ triethylamine in pyridine at room temperature for 24 hours. After removal of the pyridine, the residue was dissolved in H_2O, extracted into CH_2Cl_2 and then purified by column chromatography (silica). Recrystallisation from CH_2Cl_2/n-hexane gave the final product in 48% yield: m.p. 59-633C; ν_{max} (KBr) 3300 (NH), 1745 (ester C=O), 1665 cm^{-1} (amide C=O); s_H ($(CD_3)_2CO$) 1.99 (6H,s), 2.04 (3H,s), 3.61 (4H,t,J=6Hz), 4.20 (4H,t,J=6Hz), 6.76 (2H,d,J=9Hz), 7.40 (2H,d,J=9Hz), 8.98 (1H,br. ex.); m/z 322 (M^+).

N,N-Bis-(2-chloroethyl)-4-nitroaniline (Compound 6): This was

prepared by reaction of the corresponding diol[9] with thionyl chloride. Recrystallisation from methanol gave yellow needles: s_H ($CDCl_3$) 3.70 (4H,t), 3.86 (4H,t), 6.71 (2H,d), 8.12 (2H,d).

N,N-Bis-(2-chloroethyl)-1,4-phenylenediamine (Compound 8): This was prepared by reduction of the corresponding nitro compound following the method[5] for the nitroso derivative. It was isolated as the hydrochloride salt: s_H (D_2O) 3.79 (4H,t), 3.85 (4H,t), 6.97 (2H,d), 7.31 (2H,d).

Compounds 9-23 were prepared by reaction of the appropriate acyl chloride with 4-nitroaniline following standard procedures. All compounds were pure as determined by thin layer chromatography.

Enzyme Assay

Enzyme assays for compounds 9-23 were performed as follows[7]: 500ul of a 1mM solution of the compound was mixed with 400ul of 0.1M Tris.HCl, pH 8.6. The reaction at 303C was initiated by adding 100ul 1 unit/ml enzyme. (1 unit hydrolyses 1umole of 4-nitroacetanilide/min at 303C). The initial rate of 4-nitroaniline release was assessed against a solvent blank at 382nm.

Results

Incubation of a 1mM solution of compound 1 with 5 units of enzyme at 373C in phosphate-buffered saline resulted in virtually complete turnover of prodrug within 2 minutes, as shown by HPLC, with simultaneous release of active drug (compound 8). The reaction rate was similar over the pH range 7-8, only becoming markedly slower at pH 5 (Figure 3). Figure 4 indicates that addition of enzyme (0.4 units/ml) has virtually no effect on the hydrolysis rate of a 1mM solution of compound 2 at 373C, whereas compound 1 is virtually completely turned over within 15 minutes.

Table 1A lists the toxicities of compounds 1-8 to LS174T cells. 1 and 2 have similar toxicity, these contain potentially labile chlorine atoms, their hydrolysis rates are similar ($T_{\frac{1}{2}}$=2.5 and 2.3 hr in DMEM at 37°C, respectively) . Compound 4, which lacks labile chlorine atoms, has no detectable effect on growth. The deactivating effect of the N-acetyl group of compound 1, has little effect on its cytotoxicity, compared to that for the active drug, compound 8 ($T_{\frac{1}{2}}$=8.1min), either as a mixture or chemically synthesised.

The high sensitivity of the cells to the 4-nitroso aryl mustard (compound 5) which is stable to hydrolysis, may be due to its reduction to the highly reactive amino mustard within the cell. It was shown, by HPLC, that this occurs in the presence of NADH in cell medium (results not shown). Compound 6 which contains the strongly deactivating 4-nitro group has measurable toxicity. This result will be investigated in further experiments. A previous study[9] noted that this compound appeared to bind to plastic surfaces which reduced the efficiency of drug washout after the 1 hour drug exposure.

The JAR cell line (Table 1B) gives similar results to LS174T in that compound 3 is nearly as cytotoxic as the 4-aminoaryl mustard (compound 8).

Table 2 shows that the addition of enzyme alone up to 5 units/well has little effect on cell survival. Viability is reduced to less than 5% of control cells in the presence of 25uM compound 1 and up to 5 units/well of enzyme.

The enzymatic turnover rates for the model compounds (Table 3) indicate that the best substrates contain a N-chloroacetyl or N-acetyl group with activity diminishing rapidly if the size or

polarity of this group is altered. The model compound 12 which contains the N-trifluoroacetyl group is a poor substrate for the enzyme, as already shown for the mustard prodrug (compound 2). This is presumably due to electronic and/or steric effects of the $-CF_3$ group on the enzyme.

Discussion

This work was stimulated by the search for more potent drugs for use in antibody-directed enzyme prodrug therapy. An enzyme suitable for ADEPT therapy should not be present in the body. Acylarylamidase was selected due to its bacterial origin, although intestinal sterilisation might be required to eliminate it from the gut. In addition it seemed a suitable means of delivering prodrugs which could be converted to the extremely toxic 4-aminoaryl mustard.

The results in Table 1A are somewhat disappointing, indicating that the inclusion of an N-acyl group, which considerably stabilises the molecule, in respect of lengthening the chemical half life has little effect on the toxicity to LS174T cells. This result was also shown in JAR cells (Table 1B). The toxicity of compound 8 is, however, underestimated by a 1 hr exposure time due to its reactivity. The N-acetyl derivative is known to be biologically active against the Walker rat tumour[9], whereas the N-trifluoroacetyl and N-chloroacetyl derivatives are not active. An interesting feature of Table 1A is the relative sensitivity of the LS174T cell line to compounds 1 and 2 when compared to the much higher IC_{50} value obtained with chlorambucil (compound 7) which would be expected to have a similar mechanism of action.

It is well established that the reactivity of aromatic nitrogen mustards towards hydrolysis and alkylation is affected by

substituent electronic properties [14]. However other factors such as drug transport and metabolism may affect this relationship. For instance, the N-trifluoroacetyl prodrug (compound 2) is more lipophilic than the active drug (compound 8) and may be more readily taken up by the cell than the latter compound. For arguments concerning relative toxicities of prodrugs and active drugs it was essential to eliminate the possibility that there is a similar enzyme to acylarylamidase present in the cells. This was checked by spectrophotometric assay. Incubation of 4-nitroacetanilide with LS174T cell medium, either fresh or the cell supernatant, or a whole cell sonicate at a similar cell density to that of the cytotoxicity studies, showed that there was no formation of 4-nitroaniline. This indicated that prodrugs were not being activated by an intracellular enzyme, at least unless the putative enzyme was inactivated by cell disruption. The rate of turnover was found to be similar in the presence of fresh medium, cell supernatant or phosphate-buffered saline, rendering inactivation of the enzyme by medium unlikely.

The structure-activity relationship of model compounds in Table 3 indicate that there is a very narrow range of acyl groups which are substrates for the arylamidase enzyme, with the N-chloroacetyl analogue having the highest turnover rate within this group.

Further work is now required to prepare more hydrophilic prodrugs that are substrates and also sufficiently non-toxic.

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Table 1A. Cytotoxicity of prodrugs and 4-aminoaryl mustard in LS174T cells (1 hour exposure)

Compound		IC_{50} (uM)	IC_{90} (uM)
General structure:			
$4\text{-}R\text{-}C_6H_4\text{-}N(CH_2CH_2Cl)_2$			
	R =		
1.	$-NHCOCH_3$	3.0	11.2
	$-NHCOCH_3$ + 1U Enz	0.85	1.7
2.	$-NHCOCF_3$	1.2	10.2
	$-NHCOCF_3$ + 1U Enz	0.90	3.0
3.	$-NHCOCH_2Cl$	N/A	14.4
4.	$-NHCOCH_3$ *	>100	>100
	$-NHCOCH_3$ * + 1U Enz	>100	>100
5.	$-NO$	0.09	0.20
6.	$-NO_2$	45	320
7.	$-CH_2CH_2COOH$, Chlorambucil	38	380
8.	$-NH_2$ £	0.29	6.0

* Structure = $4\text{-}CH_3CONH\text{-}C_6H_4\text{-}N(CH_2CH_2OCOCH_3)_2$

£ Added as the hydrochloride salt

Compounds were dissolved in DMSO and diluted in medium to <5% organic solvent just prior to addition to cells.

Table 1B. Cytotoxicity of prodrugs and 4-aminoaryl mustard in JAR cells (1 hour exposure)

Compound		IC_{50} (uM)	IC_{90} (uM)
General structure:			
$4\text{-}R\text{-}C_6H_4\text{-}N(CH_2CH_2Cl)_2$			
	R =		
3.	$-NHCOCH_2Cl$	0.50	39.2
8.	$-NH_2$ £	0.22	81.0

£ Added as the hydrochloride salt

Compounds were dissolved in DMSO and diluted in medium to <5% organic solvent just prior to addition to cells.

Table 2. Effect of enzyme concentration on cell cytotoxicity of Compound 1.

Concentration	Units enzyme/Well	% Control
0uM	1	82.5
	2	93.9
	5	89.1
25uM	1	4.4
	2	3.9
	5	2.6

Table 3. Initial hydrolysis rates of 4-nitroacylanilides by the acylarylamidase enzyme.

Compound		Compound Lambda-Max	Initial hydrolysis rate (4-nitroacetanilide = 100)
	General structure:		
	NO_2-4-C_6H_4-NHCO-R		
	R =		
9.	CH_2Cl	315	119.4, 105.1
10.	CH_2Br	315	87.1, 76.7
11.	$CHCl_2$	310	45.2, 46.1
12.	CF_3	330	2.6
13.	CH_2CH_3	310	96.1
14.	$CH_2CH_2CH_3$	320	32.3, 26.1
15.	CH_2CH_2COOH	310	0, 3.7
16.	$CH_2N(CH_3)_3$ Br	310	0
17.	C_6H_5	320	0
18.	$CH_2C_6H_5$	-	0
19.	$(CH_2)_5CH_3$	320	2.8
20.	$(CH{=}CH)CH_3$	310	9.5, 12.5
21.	$(CH{=}CH)C_6H_5$	310	5.6, 0
22.	$(CH_2)_2CONH$-4-C_6H_4-NO_2	-	0
23.	$(CH_2)_4CONH$-4-C_6H_4-NO_2	310	0

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Legend to Figures

Figure 1:

The N-acetylaryl mustard prodrug (compound 1) and its amino mustard product (compound 8) generated by acylarylamidase.

Figure 2:

HPLC chromatograms of N-acetylaryl mustard prodrug (compound 1) A: in the absence & B: in the presence of 0.4U/ml acylarylamidase enzyme with release of 4-aminoaryl mustard (compound 8).
HPLC chromatograms of N-trifluoroacetylaryl mustard prodrug (compound 2) C: in the absence & D: in the presence of 0.4U/ml enzyme.
Compounds were incubated at 37°C for 15 minutes.

Figure 3:

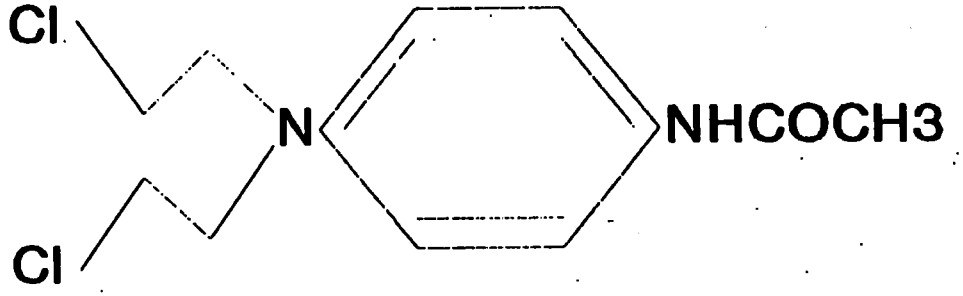
Initial turnover of 1mM solution of compound 1 by acylarylamidase enzyme (0.25U/ml) at pH 5.0 - 8.0. Samples were analysed after 5 minutes at 37°C by HPLC.

Figure 4:

Effect of acylarylamidase enzyme on 1mM solutions of compound 1 (⊞) and compound 2 (◇) in fresh DMEM at 37°C. Solid line - no enzyme, dotted line - 0.4 units/ml.

PRODRUG

T1/2 = 2.5 hr



Acylarylamidase

DRUG

T1/2 = 8.1 min

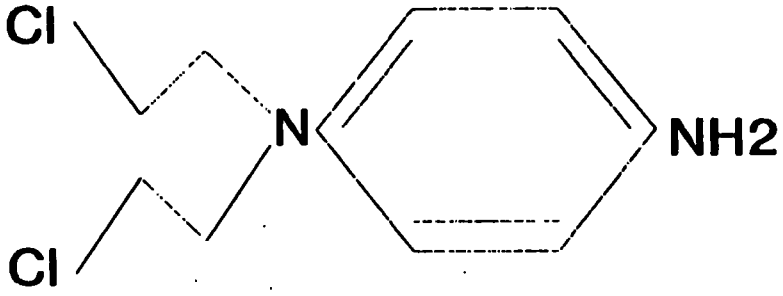
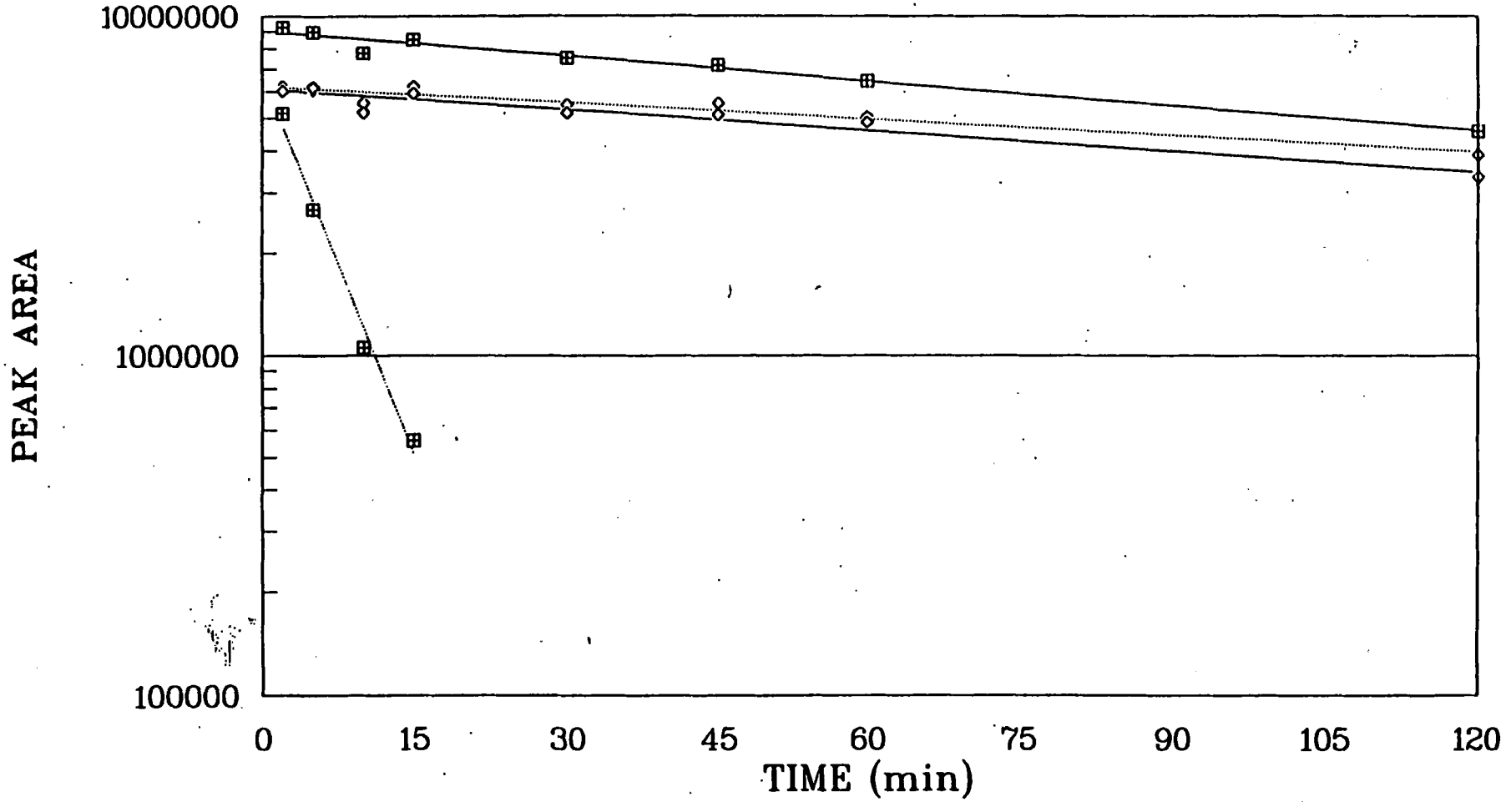


Figure 1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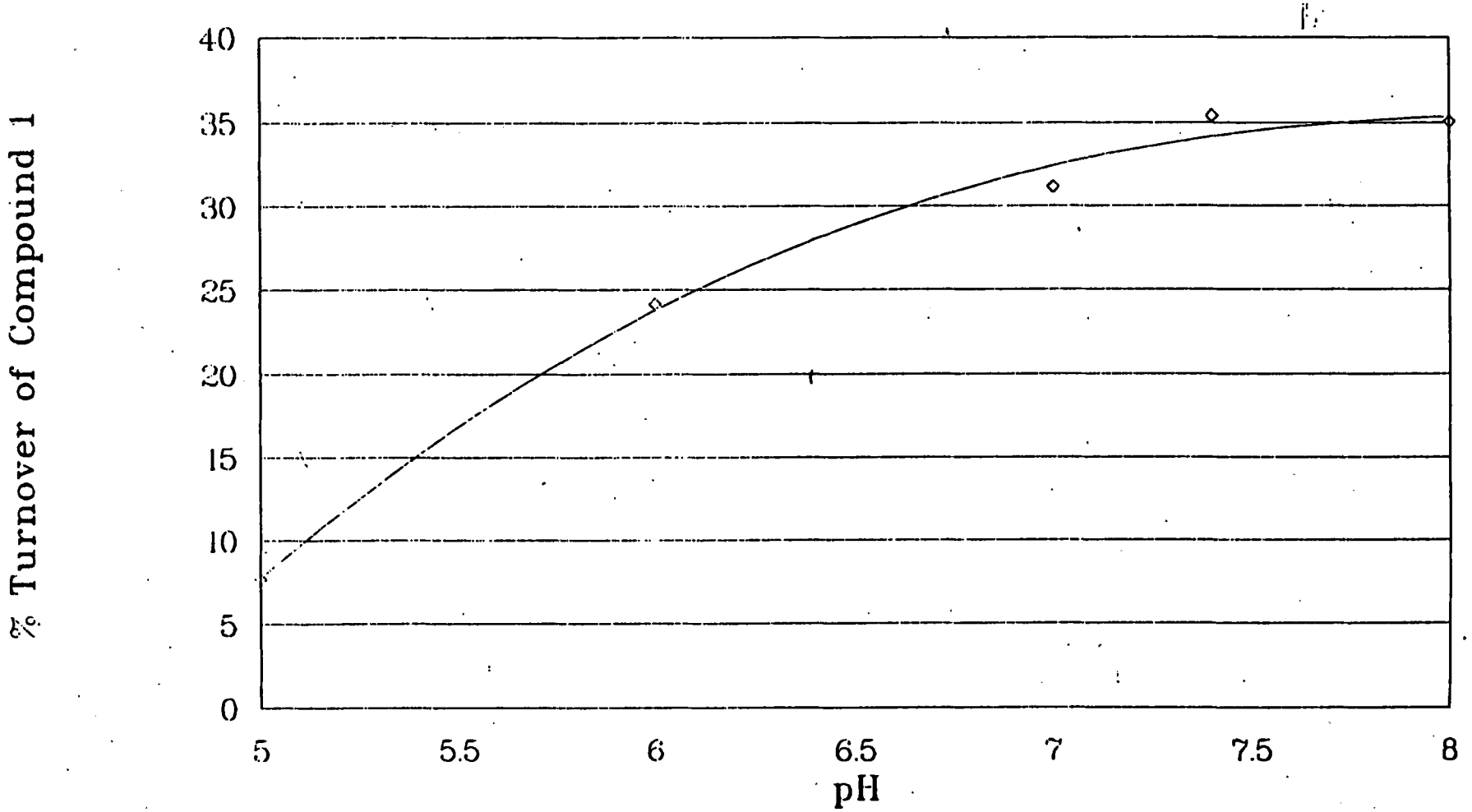
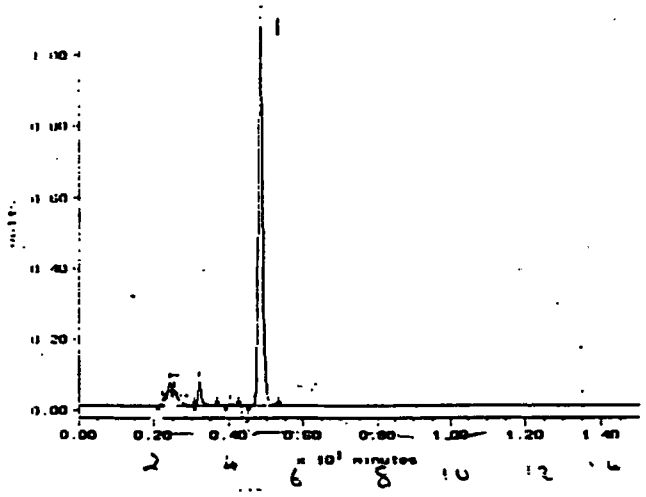
1000000
100000
PEAK AREA
0
15
30
45
60
75
90
105
120
TIME (min)


% Turnover of Compound 1
40
35
30
25
20
15
10
5
0
5
5.5
6
6.5
7
7.5
8
pH

(Copy for Medical Illustration)

A



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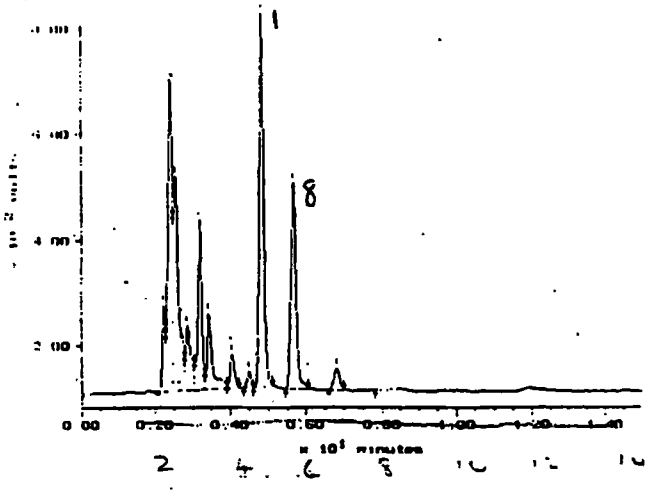

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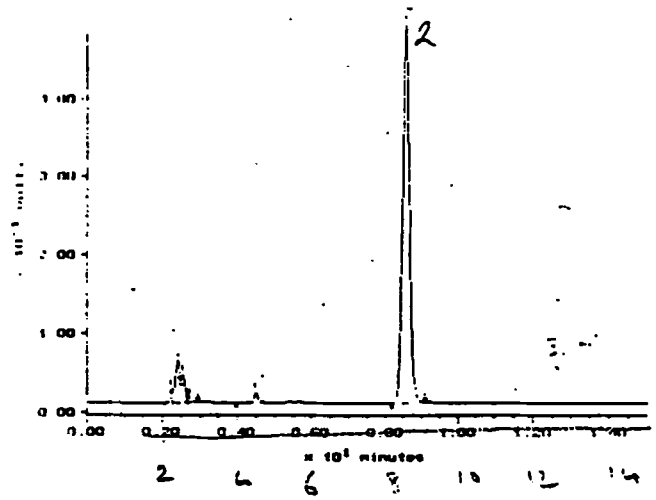

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